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Testing theories for ecological restoration: effects of plant-plant interactions and plant order of arrival on assembly and on above- and belowground productivity

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Emanuela Wehmuth Alves Weidlich

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Author’s address:

Leuphana University, Institute of Ecology

Scharnhorststraße 1, 21335 Lüneburg, Germany

e-mail: emanuela.weidlich@leuphana.de

“Palavra puxa palavra, uma ideia traz outra, e assim se faz um livro, um governo, ou uma nação, alguns dizem mesmo que assim é que a natureza compõe suas espécies.”

Machado de Assis

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TABLE OF CONTENT

SUMMARY 02

CHAPTER 1 04
General introduction

Papers 1 to 5:

CHAPTER 2 (paper 1) 22
The importance of being first: exploring priority and diversity effects in a grassland field experiment. Weidlich *et al.* (2017). Frontiers in Plant Science.

CHAPTER 3 (paper 2) 42
Priority effects caused by plant order of arrival affect belowground productivity. Weidlich *et al.* (2017). Journal of Ecology (in press).

CHAPTER 4 (paper 3) 59
Methane yield of biomass from extensive grassland is affected by compositional changes induced by order of arrival. Popp *et al.* (2017). *GCB Bioenergy*.

CHAPTER 5 (paper 4) 75
Neighbourhood stories: role of neighbour identity, order of arrival and spatial location in legume and non-legume interactions. Weidlich *et al.* (Submitted to Plant and Soil).

CHAPTER 6 (paper 5) 94
Accuracy and bias of methods used for root length measurements in functional root research. Delory *et al.* (2017). Methods in Ecology and Evolution.

CHAPTER 7 117
Synthesis and Conclusions

REFERENCES 126

CURRICULUM VITAE 140

APPROVAL OF OWN RESEARCH 143

SUMMARY

European species-rich grasslands, which provide many ecosystem functions and services, are threatened both by land use intensification as well as land abandonment. The studies shown in this thesis tested the possible use of ecological knowledge to ensure hay productivity whilst maintaining diversity of grasslands, with a view to informing ecological restoration. The overall approach was to understand interactions between plants, to study diversity effects on productivity, and mainly investigate how plant functional groups that arrive first in the system can create priority effects that influence community productivity both above- and belowground.

A grassland field experiment was established and monitored for four years, in order to verify the effects of manipulating the order of arrival of different plant functional groups, as well as the sown diversity level on productivity and methane yield. The overall findings were: a) sowing legumes first created priority effects aboveground (higher biomass) and belowground (lower root length), plants invested less in roots and more in shoots, b) priority effects were more consistent below than aboveground, c) sown diversity did not affect aboveground biomass, d) the order of arrival treatments indirectly affected methane yield by affecting the relative dominance of plant functional groups.

Since we lack information on how legumes and non-legumes interact spatially belowground, (particularly related to root foraging) a controlled experiment was performed, using two grass species and one legume. The identity and location of the neighbours played a role in interactions, and the order plants arrived modulated it. When the focal species (grass) was growing with a legume it generally equated to the same outcome as not having a neighbour. Roots from the focal species grew more toward the legume than the grass neighbour, indicating a spatial component of facilitation.

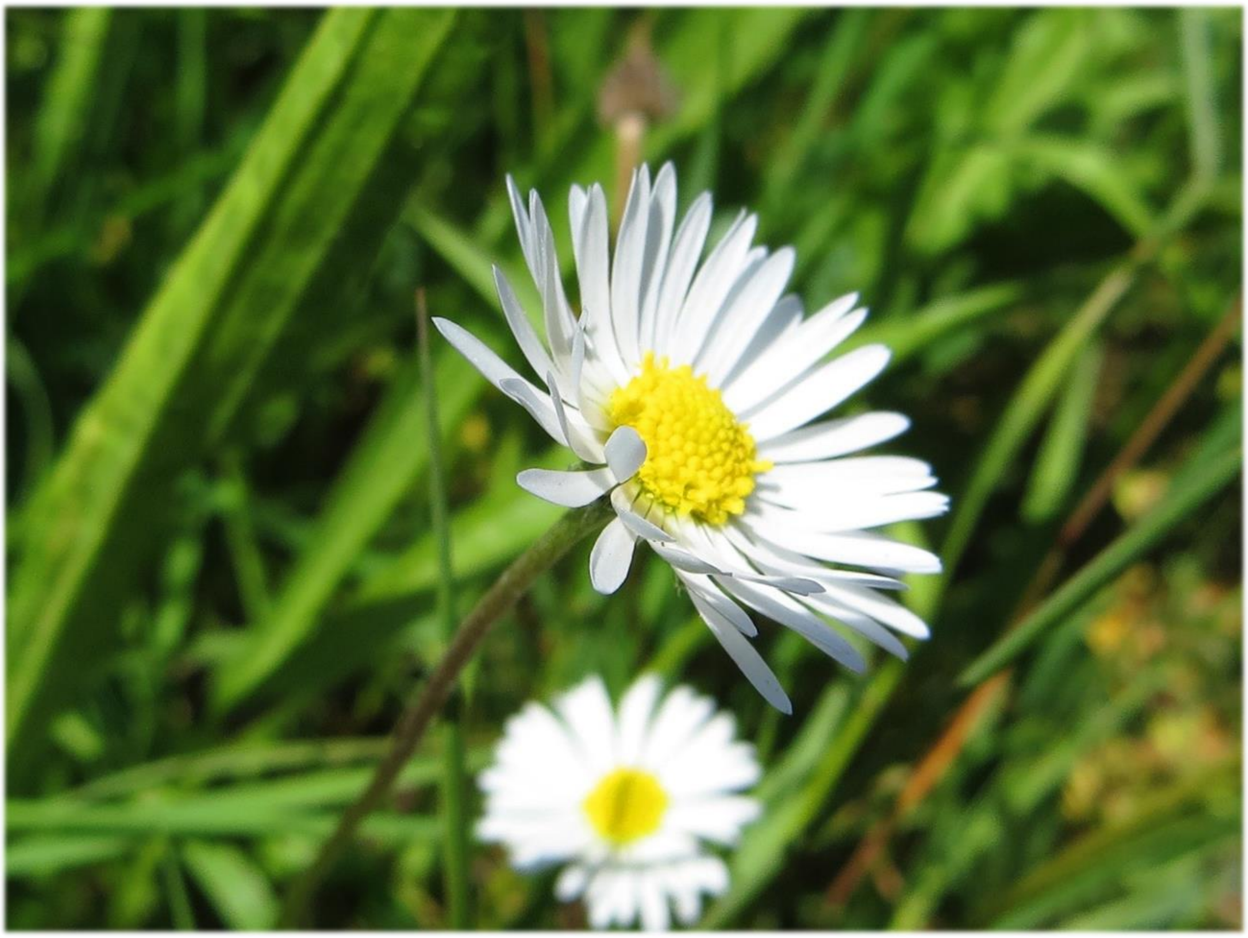
Since these studies involved root measurements, a method study was also conducted to verify how comparable and accurate are root length estimates obtained from different techniques. Results showed that the use of different methods can lead to different results,

the studied methods did not have the same accuracy, and the automated methods can underestimate the root length.

Overall, these results allow to conclude that different groups of plants arriving before others affected above and belowground biomass, roots may be key drivers during the creation of these priority effects, and interaction outcomes between plants depended on neighbour identity and location, modulated by the order they arrive in. Our results suggest that we can use priority effects by sowing different species or plant functional groups at different time to steer a community to a desired trajectory depending on the restoration goal (such as increasing biomass whilst maintaining diversity). However, there is a need to test contingency, potential, and long term impacts of such possible tools for restoration.

CHAPTER 1

General Introduction



PLANT-PLANT INTERACTIONS IN COMMUNITY ASSEMBLY

Understanding community assembly has been a primary goal of ecologists for over a century. Despite the increase of studies addressing what governs community development, the question on how communities come to be the way they are remains unclear. In addition, how organisms interact, and how these interactions affect community structure and development need better understanding. One of the main motivations for understanding what governs assembly is for its potential application in restoration ecology (Temperton et al. 2004; Martin and Wilsey 2013). This is important, since the more we understand what regulates assembly, the more information we will have on how to return a degraded area to a functioning ecosystem.

Theories about how communities are structured were first introduced in the early 20th century by Clements and Gleason, who used the term "succession". In the stochastic model from Gleason, community composition and structure are a random process that depend only on dispersal and the order in which species arrive (Gleason 1926). Clements' deterministic theory stated that organisms successively establish in a system after a major disturbance and develop until a stable equilibrium, the so-called climax community (Clements 1936). Later, a new model called alternative stable states (Sutherland 1974) and more recently named alternative states (since they can be stable or transient, Fukami & Nakajima 2011) is an intermediate between Clements and Gleason models. Here communities are structured and at the same time restricted to a certain extent by interactions between organisms and their environment, but can develop towards different stable or transient states. Which alternative states occur depends on the interactions between organisms as well as climate and soil drivers. These stable states are often not just a result of abiotic factors, but often very much driven by biotic interactions between species and the order they arrive in the system.

Succession and assembly are related. Young, Chase & Huddleston (2001) compared and contrasted the primary concept of community succession and the more recent ideas of community assembly (Drake 1990) in the context of the development of ecological communities and their relevance for ecological restoration. Succession is described as the orderly and predictable way in which communities establish, and refers to an endpoint, a single climax. Assembly refers to development, which is driven by a random variation in species colonization and persistence in the community, focusing in interactions between organisms and the pathway the community can take in response to these interactions. **This thesis concentrates in plant community assembly, since the main focus was to evaluate if the order of arrival of different plant functional groups can create alternative states, changing the trajectory of a community as it assembles.**

PRIORITY EFFECTS

Assembly can be modulated by the organisms which arrive first. This is called priority effects, in which organisms that first arrive at a site can significantly affect the establishment, growth, or reproduction of the species arriving later, thus influencing further assembly (Vannette & Fukami 2014; Vaughn & Young 2015; Temperton *et al.* 2016). Thereby, priority effects can affect the community structure, as well as ecosystem functioning and services. In order to broadly clarify the concept, Table 1.1 lists several definitions of priority effects taken from papers in which it was experimentally tested. Studying priority effects can be broadly defined as the evaluation of the effects order of arrival of plant species or functional groups has on measured variables, such as biomass, cover, diversity and abundance. There is a trend of describing priority effects as a negative effect in the community, taking into consideration only how the first arrivals compete with the species arriving later. However, the first plants that arrive in the system can also positively affect the trajectory of the community by facilitation. Thus, priority effects can be negative or positive, being related with competition and facilitation, which will be discussed later in this introduction.

Some authors also include diversity as a type of priority effect, considering that a different initial composition, such as sowing higher (vs. lower) diversity seed mixtures, has a sustained positive effect on ecosystem functioning over years (Corbin & D’Antonio 2004; Fukami *et al.* 2005; Lulow 2006; Carter & Blair 2012; Plückers *et al.* 2013; Roscher *et al.* 2014). However, in this thesis priority effects are defined more narrowly as only those related to timing/order of arrival, and not when addressing diversity.

Author	Concept of priority effects
Quinn & Robinson 1987; Abraham, Corbin & D'Antonio 2009; Collinge & Ray 2009; Del Moral, Sandler & Muerdter 2009; Woodcock, McDonald & Pywell 2011; Wainwright, Wolkovich & Cleland 2012; Young *et al.* 2014; Fukami 2015; Vaughn & Young 2015; Brandt *et al.* 2016; Dhami, Hartwig & Fukami 2016; Sarneel *et al.* 2016; Stuble & Souza 2016	Order/timing of arrival, emergence, colonization, and establishment, affect the establishment of later-arriving species.
Körner *et al.* 2008; Griffith & Loik 2010; Stevens & Fehmi 2011; Cleland, Esch & McKinney 2015; Helsen, Hermy & Honnay 2016	Early colonizing species. Earlier planting. "Who comes first". Unequal germination time. Earlier growth/establishment/ arrival.
Grman & Suding 2010	When earlier growing species affect the establishment, growth, or reproduction of later growing species can lead to long lasting differences in species dominance.
Halpern *et al.* 2016	Initial colonists pre-empt space or resources from later arrivals, shaping the outcome of community reassembly
Kardol, Souza & Classen 2013	Where early-arriving species influence the establishment and growth of later arriving species.
Ladd & Facelli 2008	Subtle changes in timing of emergence. A form of positive feedback that results from one species changing the abiotic environment so that it favor con-specifics, while at the same time, disadvantages other species.
Lulow 2006	Differences in arrival order resulting in community states that differ in species composition or abundance.
Martin & Wilsey 2012	When an early-establishing species is able to attain large size before its competitor arrive, which enables it to out-compete later-arriving species.
Mason, French & Jolley 2013	The first species establishes and changes the abiotic environment, influencing site favorability for subsequent immigrating species.
von Gillhaussen *et al.* 2014	When one (or more species) already present in a habitat thereby affects the success of later species, and this effect can be negative, neutral or positive.

Table 1.1. Concepts of priority effects given by different authors.

Connell & Slatyer (1977) were the earliest ecologists discussing the role of early colonizers. They described three mechanisms which would produce the sequence of species in succession, stating that the first colonists at a disturbed site can have a positive (facilitative), negative (inhibition) or neutral (tolerant) effect on the later ones. A decade later, Quinn & Robinson (1987), in studies with Californian grasslands, used the term priority effect for the first time in order to justify their findings, reporting that the species which established earlier suppressed the later arrivals. Later, studying community assembly mechanisms in freshwater controlled ecosystem (using from bacteria to invertebrates), Drake (1991) performed the first experiment testing priority effects and found that species dominance was determined by the species that first arrived in the assembly sequence, what he called priority effect.

Since then, the term priority effects has been used to discuss and justify findings such as establishment in restoration sites (Trowbridge 2016), community dispersion (Houseman *et al.* 2008), initial species composition in succession (Rebele 2008), restoration methods (Klimkowska *et al.* 2010; Pfeifer-Meister *et al.* 2012), weed control (Milchunas *et al.* 2011), competitive interactions after disturbance (Moore & Franklin 2011), natural regeneration (Norden *et al.* 2011), grazing managements (Woodcock *et al.* 2011), and plant traits in community assembly (Helsen, Hermy & Honnay 2012). But only in the last 15 years have priority effects been explicitly tested in experiment designs. Part of these studies used native species in controlled experiments (Young *et al.* 2001; Ejrnæs, Bruun & Graae 2006; Körner *et al.* 2008; Moore & Franklin 2012; Kardol *et al.* 2013; von Gillhaussen *et al.* 2014) or field experiments (Lulow 2006; Collinge & Ray 2009; Schantz, Sheley & James 2015; Helsen *et al.* 2016). The majority of the studies were interested in the effects of order of arrival between exotic and native species in controlled experiments (Abraham *et al.* 2009; Grman & Suding 2010; Stevens & Fehmi 2011; Byun, de Blois & Brisson 2013; Mason *et al.* 2013; Orloff, Mangold & Menalled 2013; Aschehoug *et al.* 2014; Ulrich & Perkins 2014; Burkle & Belote 2015; Wilsey, Barber & Martin 2015) or field experiments (Corbin & D'Antonio 2004; Daehler & Goergen 2005; Martin & Wilsey 2012; Wainwright *et al.* 2012; Cleland *et al.* 2015; Vaughn & Young 2015; Young & Veblen 2015). In order to study priority effects by manipulating different plant functional groups, Körner *et al.* (2008) sowed either legumes, non-legume forbs, or grasses first and the other two groups three weeks later. They found strong priority effects of sowing legumes first on both, aboveground and belowground community productivity, even after two growing seasons. The same aboveground pattern was found in a controlled experiment by von Gillhaussen *et al.* (2014).

Grman and Suding (2010) suggested that priority effects arise from asymmetric competition and soil legacies. Asymmetric competition occurs when interacting plants have different sizes and hence their competitive interaction is not balanced and the dominant plant has a much higher chance of outcompeting the smaller one. Soil legacies occur when the presence of a specific plant species affects the microbial community which affects the plant itself or other individuals. Fukami (2015) proposed that priority effects happen by two mechanisms, which he named niche pre-emption and niche modification. In niche pre-emption the first arrival species reduce the amount of resources available to other species, resulting in inhibitory effects. In niche modification, the species that first arrive change the niches available, consequently modifying the identity of the species able to arrive later. In this case, priority effects could be inhibitory or facilitative.

An important theory related to assembly and priority effects is the filter theory. It asserts that species are sorted according to their ability to survive biotic and abiotic filters (Keddy 1992; Diaz, Cabido & Casanoves 1998; Temperton *et al.* 2004). A species pool will be affected by abiotic (e.g. temperature, soil properties) and biotic (e.g. competition, predation) filters which only allow certain species “to pass through the mesh” and establish. The order of arrival of species during assembly (priority effects) is an example of biotic filter, which together with other filters can influence assembly and/or the success of restoration (Temperton *et al.* 2004).

Priority effects are at the heart of the studies developed in this thesis. The main goal was to understand how manipulating the order of arrival of different groups of plants affect the trajectory of a community.

BIODIVERSITY-ECOSYSTEM FUNCTIONING

The relationship between biodiversity and ecosystem functioning has become an important topic in plant ecology in the last decades, together with the large impact of humans in the ecosystems around the world (Loreau *et al.* 2001; Tilman *et al.* 2001; Balvanera *et al.* 2006;

Flombaum & Sala 2008). Classical biodiversity-ecosystem functioning (BEF) experiments, such as the Jena experiment in Germany (Marquard *et al.* 2009; Ravenek *et al.* 2014; Roscher *et al.* 2014) or the Cedar Creek study site in the United States (Tilman *et al.* 2001; Isbell *et al.* 2011) have found clear evidence that biodiversity has positive effects on ecosystem functioning and services. A higher diversity of plant species affects positively many aspects of ecosystem functioning, for example by increasing productivity (Flombaum & Sala 2008), soil fertility (Dybzinski *et al.* 2008), accelerating species development (Kirmer, Baasch & Tischew 2012), or reducing invasibility (Hector *et al.* 2001; Kennedy *et al.* 2002; van Ruijven, De Deyn & Berendse 2003). These positive biodiversity effects on biomass production found in BEF experiments (Hector & Bagchi 2007; Picasso *et al.* 2011) can even positively influence bioenergy production. Khalsa *et al.* (2014) found an increase in methane yield with higher biomass induced by an increase in species richness. Studies have found a correlation between biogas yield and functional group composition, as well as a key role for legume and grass composition (Khalsa *et al.* 2014; Van Meerbeek *et al.* 2015). However, even though there are studies testing diversity effects on methane yield, we lack knowledge on how a combination of diversity and priority effects may perform in this respect.

This response to diversity can be explained by the *insurance hypothesis* (Yachi & Loreau 1999), where diversity insures ecosystems against declines in their functioning because a higher number of species provides greater guarantees that some will maintain functioning even if the others fail. This theory is a fundamental principle in understanding the long-term effect of biodiversity on ecosystem processes.

Aiming to test whether positive diversity effects found in biodiversity experiments could be applied to the restoration of grasslands, Bullock *et al.* (2001, 2007) sowed either low or high diversity mixtures (all species sown at the same time) in UK grassland restoration ex-arable sites and followed their development over eight years. They found (in 17 different restored calcareous grasslands) that sowing high diversity seed mixtures led to both higher aboveground productivity and diversity in comparison to grasslands which were sowed with low diversity seed mixtures. Even though the treatments were under the same environmental conditions, the initial sowing affected the development of the community. Bezemer & Van Der Putten (2007) performed an experiment sowing either zero, four or fifteen species of plants onto ex-arable land then followed the dynamics of the system in terms of species turnover, productivity, temporal stability and diversity. Both studies found long-lasting diversity effects on further assembly. Flombaum & Sala (2008) removed species to create a plant species diversity gradient in the Patagonian steppe and found that aboveground net primary production increased with the number of plant species. **The studies about priority effects together with these ones about diversity, inspired the establishment of the *Priority effect experiment* which was the main object of study from this thesis, where priority and diversity effects were combined experimentally for the first time. We were mostly interested to know how much diversity and priority effects influence biomass productivity above and belowground, as well as methane yields.**

COMPETITION AND FACILITATION

Competition "is any use or defense of a resource from an individual which reduces the availability of this resource to another individual" (Huston & Smith 1987; Goldberg & Barton 1992; Bengtsson, Fagerstram & Rydin 1994; Callaway & Walker 1997). Since competition can determine species diversity through species interactions (Chesson 2000), it has been measured in several ways to understand interactions between plants and its consequences for community assembly. The first study which experimentally tested competition (Tansley 1917) was conducted by sowing two different forb species and showed that when grown alone, each species could survive, but when grown together, soil type determined which would survive. Gause (1934) introduced the competitive exclusion theory, which stated that two species with the same niche (environmental conditions that allow a species to survive) cannot coexist. Niche overlaps can lead to niche partitioning and to competitive coexistence, while strong niche overlap lead to competitive exclusion. The contemporary coexistence theory suggests that species coexist depending on niche and fitness differences (Chesson 2000; HilleRisLambers *et al.* 2012; Valladares *et al.* 2015).

In ecology, for a long time it was assumed that when plants interact, they always compete, meaning that interactions would always be negative. However, plants can also positively interact, known as facilitation, which can happen simultaneously with competition (Callaway 1995; Callaway & Walker 1997). While competition is a well-studied topic and was experimentally tested for the first time in the beginning of the last century (Tansley 1917), facilitation is overlooked and less considered in plant ecology studies (Brooker *et al.* 2008;

Wright *et al.* 2017). To explain the mechanisms of succession Connell & Slatyer (1977) discussed facilitation for the first time, but the concept started to be clearer when Bertness & Callaway (1994) and Callaway (1995) highlighted that plants also interact positively, increasing research on this topic (Brooker *et al.* 2008; Holmgren & Scheffer 2010; Bessler *et al.* 2012; Gagliano & Renton 2013). This is very important to the study of plant-plant interactions, since facilitation may determine the community structure, allow species coexistence, increase diversity and productivity, and most importantly, influence species distribution driving communities (Callaway 1995). Thereby, a community is the result of negative and positive interactions.

As a common example of facilitation, nitrogen fixing plants (legumes from the Fabaceae family) have been reported to show positive effects on their neighbours (Li *et al.* 2003; Temperton *et al.* 2007; Hauggaard-Nielsen *et al.* 2008; Bessler *et al.* 2012; Frankow-Lindberg & Dahlin 2013). Named as nitrogen (N) facilitation, it works via an increase in the availability of N for neighbours of legumes (Schenk 2006; Temperton *et al.* 2007). Mechanisms of N facilitation include N transfer and N sparing. The first is described as direct N transfer from the legume to neighbours (either via mycorrhizae or root exudation), while the second occurs when non-legume neighbours profit from the spare N available in the soil that legumes are not taking up since they mainly rely on N fixed from the atmosphere (Temperton *et al.* 2007; von Felten *et al.* 2009). Several experiments have shown the positive effects of including legumes in sowing mixtures. In a biodiversity ecosystem functioning experiment Bessler *et al.* (2012) found higher N uptake in grasslands with legumes than without. Frankow-Lindberg & Dahlin (2013) found in a grassland field experiment that non-legumes benefited from the presence of legume-species. Hauggaard-Nielsen & Jensen (2005) highlighted the role of roots in facilitative interactions in intercropping systems. Temperton *et al.* (2007) showed the facilitative role of legumes in the Jena experiment. The beneficial effects of intercropping with a legume species in agriculture has also been tested aiming to improve agricultural management (Li *et al.* 2003; Fan *et al.* 2006; Hauggaard-Nielsen *et al.* 2008; Ramirez-Garcia *et al.* 2014; Nabel *et al.* 2016).

Facilitation and competition can be modulated by the order in which species arrive in the system. In addition, how plants interact (positively or negatively) with their neighbours, can depend on neighbour identity (Semchenko, Saar & Lepik 2014), its location, as well as the time they arrived in. Although facilitative effects of having legume neighbours are well studied, a better mechanistic understanding of how legumes interact with non-legumes in terms of root distribution is needed. Studies manipulating the order of arrival of different plant functional groups found that when legumes were sown first, the biomass of the community was higher than when grasses or forbs were sown first, showing the role of facilitation by nitrogen-fixing plants in assembly (Körner *et al.* 2008; Von Gillhaussen *et al.* 2014). Since facilitation can drive a community, understanding how plants positively interact can be a powerful tool to steer a community to a desired state, such as restoration outcomes. **In the studies reported in this thesis, we were particularly interested in investigating how priority effects can promote positive effects in community composition and productivity. More specifically whether nitrogen-fixing plants arriving before other groups of plants would positively affect community assembly, and on how plants interact belowground depending on the neighbour identity and location, as well as the order of arrival.**

SCOPE OF THIS THESIS - SUMMARY OF THE INCLUDED STUDIES AND MAIN QUESTIONS

The main goal of this thesis was to understand interactions between plants, to study diversity effects on productivity, and mainly investigate how plant functional groups that arrive first create priority effects that can influence the trajectory of a community in order to inform restoration. Overall, a grassland field experiment was monitored (*Priority effect experiment* - Box 1) and a controlled experiment was performed (*Rhizotron experiment* - Box 2) by measuring plant above and belowground parameters in order to be able to answer the main following questions:

a. What are the effects of manipulating the order of arrival of different plant functional groups and the diversity level on aboveground biomass and community composition **(chapter 2)**?
b. What are the effects of manipulating the order of arrival of different plant functional groups on belowground productivity **(chapter 3)**?

c. What are the effects of manipulating the order of arrival of different plant functional groups and the diversity level on aboveground biomass productivity and methane yield **(chapter 4)**?
d. Is above and belowground plant performance affected by having a neighbour, as well as by the identity of the neighbour, its spatial location and the order they arrive **(chapter 5)**?
e. How comparable and accurate are the root length estimates given by manual and automated methods **(chapter 6)**?

These questions are answered in chapters 2 to 6, where each chapter presents a scientific paper (Fig. 1.1). The *Priority effect experiment* (Box 1) was established in 2012 and monitored until 2015. It was a unique experiment, since it combined assembly and diversity in the same experimental set up. We were interested to find which plant functional group sown first and which diversity level promote higher biomass, as a way to motivate farmers to sow diversity thus increasing productivity as well as willingness to maintain or restore species-rich grasslands. We collected shoot biomass and cover data every year. In addition, roots were collected in the first and third years in a sub-set of plots. In **chapter 2** we studied whether diversity and priority effects affected the aboveground biomass, abundance and species composition of the *Priority effect experiment*. This topic is expanded in **chapter 3**, reporting the belowground results from this field experiment, where we investigated whether priority effects would affect belowground productivity. **Chapter 4** shows the results of a collaboration with UFZ Leipzig, where we measured biomass production and methane yield in the second year of the *Priority effect experiment*.

Since we were also interested to know more closely how legume species interact with non-legumes species we performed the *Rhizotron Experiment* (Box 2), the results of which are shown in **chapter 5**. Here we investigated the effects of neighbour identity, spatial location, and plant order of arrival on root and shoot performance.

While working with the roots collected in the *Priority effect experiment*, we faced the question on whether we could compare root length estimates obtained from different techniques, and which methods we should use to analyse them in order to obtain accurate root length estimates. It gave us the opportunity to compare different methods, and the results of this comparative study are reported in **chapter 6** (a method paper).

Finally, in **chapter 7** the results from the five manuscripts described in the previous chapters are synthesized and contextualized. The importance of our studies and the results found are discussed, as well as how they contributed to the study of priority effects. Chapter 7 also points out how our findings are relevant to ecological restoration.

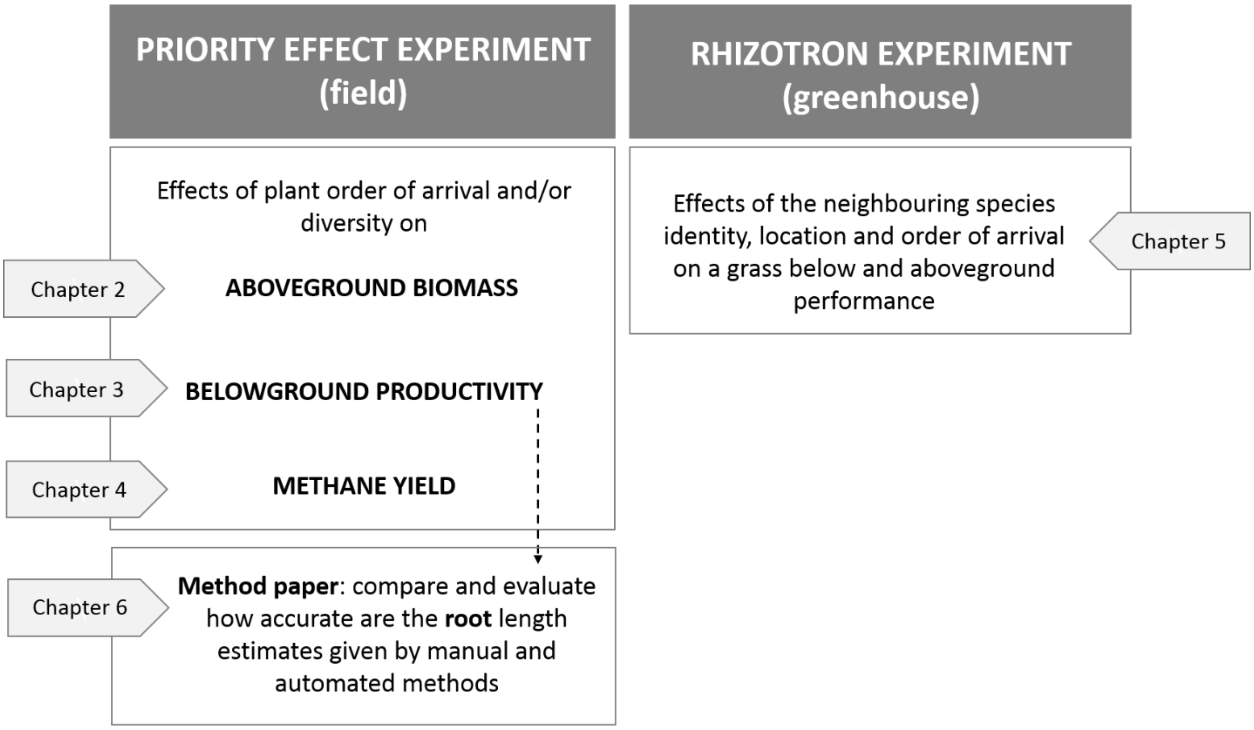


Figure 1.1. Diagram representing how the studies are divided in each chapter.

BOX 1. ***PRIORITY EFFECT EXPERIMENT*** **(FIELD)**

Most of the data on which this thesis is based were collected in the *Priority effect experiment*. More detailed information is provided in chapters 2 and 3, which contains the above- and belowground results.

The experiment was located on an ex-arable field southeast of Jülich (Germany), and was set up on two areas (A and B - Fig. 1.2) reflecting two different soil types. The main factors of the experiment were the sown diversity (2 levels: high or low diversity), the order of arrival of three different functional groups (4 levels: grasses, forbs or legumes sown first and all plant functional groups sown at the same time) and the soil type (2 levels: area A and area B). Areas A and B had exactly the same treatment factors with four replicates each resulting in a total of 64 plots. The high diversity treatments were composed by 21 species and the low diversity treatments by 9 species (with 7 or 3 species belonging to each functional group, respectively). The order of arrival treatment was created by sowing the species of one plant functional group (or the control plots) six weeks before the species from the other plant functional groups (Fig. 1.3). It resulted in four order of arrival treatment: forbs-first (F-first), grasses-first (G-first), legumes-first (L-first) and control (all plant functional groups sowed at the same time). We sowed plant functional groups (instead of species) to provide functional diversity (Lavorel *et al.* 1997; Pillar 1999). BEF experiments have shown that plant functional group identity and combinations can significantly drive ecosystem functions. In addition, Körner *et al.* (2008) found that manipulating the order of arrival of different plant functional groups significantly affected biomass production.

Table 1.2 shows all the measurements taken in the *Priority effect experiment* over the four years, as well as the manuscripts in where the collected data was analysed and reported. Aboveground biomass and species composition (either by cover or biomass per species) were collected from 2012 to 2015. Root material was collected in the first and third year of the experiment. Standing root length was obtained by collecting soil cores (Fig. 1.4a) and fine root length density by inserting mesh bags (Fig. 1.4b) in the plots, using the Ingrowth core method. A combined total of 288 root samples per studied year were collected and roots were washed and analysed in order to obtain root length density and dry weight (Fig. 1.4c).

Figure 1.2. Aerial image showing the distribution of the plots on area A and B (with two different soil types, named area A and B) in the *Priority effect experiment* in Jülich, Germany, summing a total of 64 plots of 4 m x 4 m. Eight extra plots can be seen in the figure, where anything was sown, as well as smaller monoculture plots (neither data is reported here).

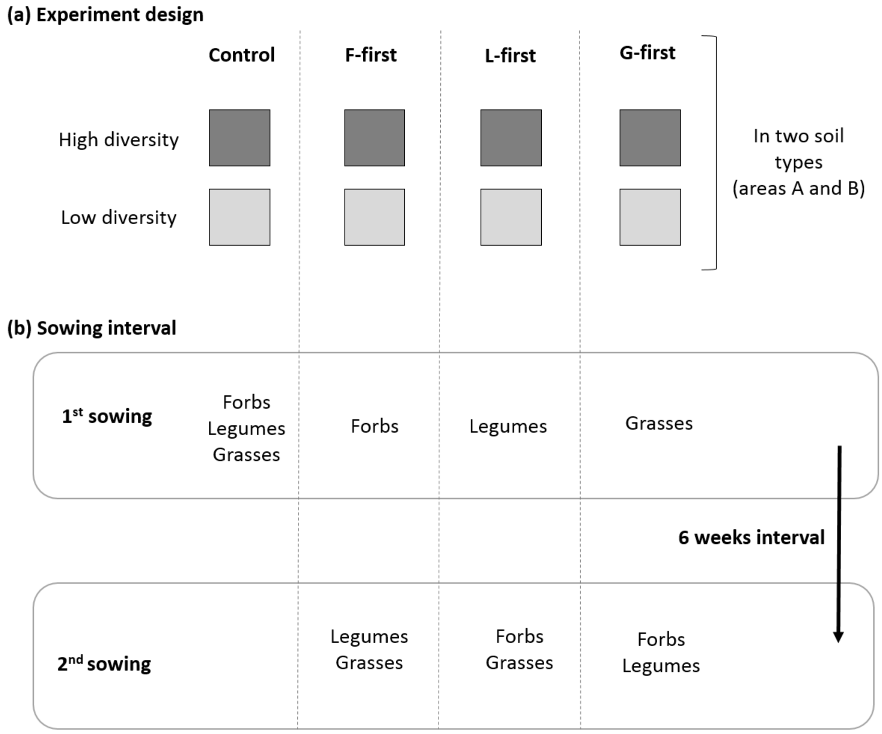


Figure 1.3. Diagram showing the experiment design (a), and how the sowing interval (b) was conducted in order to establish the order of arrival treatments. The main factors were sown diversity, order of arrival, as well as soil type (2 levels * 4 levels * 2 levels * 4 replicates = 64 plots).

Table 1.2. Data measured in the *Priority Effect experiment* from 2012 to 2015, and the number of the chapter in which the data is presented.

Data	**Year**			
	2012	**2013**	**2014**	**2015**
Plant cover	2	2		2
Total aboveground biomass	2	2 4	2	2
Aboveground biomass per species			2	
Standing and fine root length density	3 6		3 6	
Soil properties	2 3		2 3	

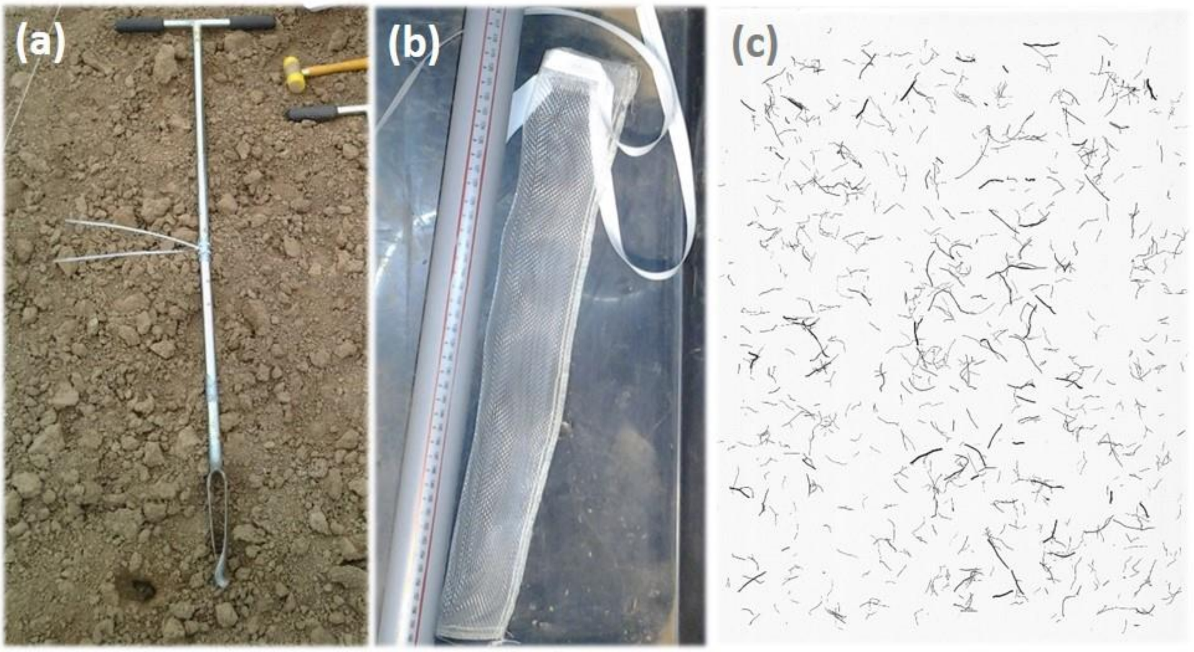


Figure 1.4. Roots collected in the experiment in 2012 and 2014: standing root collected with normal soil cores (a), fine roots were obtained with the ingrowth core method, by using the mesh bags where fine roots were allowed to grow inside, and PVC tubes to insert the bags (b), roots scanned to obtain root length (c). More details in chapters 3 and 5.

BOX 2: *RHIZOTRON EXPERIMENT* (CONTROLLED)

A brief explanation about the *Rhizotron experiment* is described here. More detailed information, including references for the methods used here, are explained in chapter 5.

This four-week controlled experiment was conducted from October to November 2013. Rhizotrons (Fig. 1.5a, b) were used to be able to see the roots, and sowed maize alone or together with one or two other species, wheat and/or clover. To be able to distinguish the roots in the soil, genetically transformed maize was used including the gene for *gfp* (Green Fluorescence Protein) and genetically transformed wheat including the gene for *rfp* (Red Fluorescence Protein). Clover was grown as wild type, the roots of which could be distinguished from the other two species with the *gfp* and *rfp* (coloured roots).

We aimed to verify whether maize above and belowground performance was affected by having a neighbour, as well as by the identity of the neighbour, its spatial location and the order they arrive (priority effects). The rhizotron area was divided into three compartments with maize sowed in the middle (focal plant), and a legume and non-legume neighbour sowed in left and right side, at different spatial locations.

Since we wanted to identify the roots from different species, we used an imaging system for identifying roots that can emit green and red fluorescence from transgenic maize and wheat respectively. With proper lighting and filtering, only roots of plants expressing the *gfp* or *rfp* were visible, allowing us to register each root position and trace it on each measured day, drawing them manually on the transparent interface of the rhizotron (Fig. 1.5b). Images of roots were acquired with a conventional camera, and roots previously drawn on the transparent interface of the rhizotrons were digitally re-drawn using a software and a computer mouse graphics table (Fig. 1.5c).

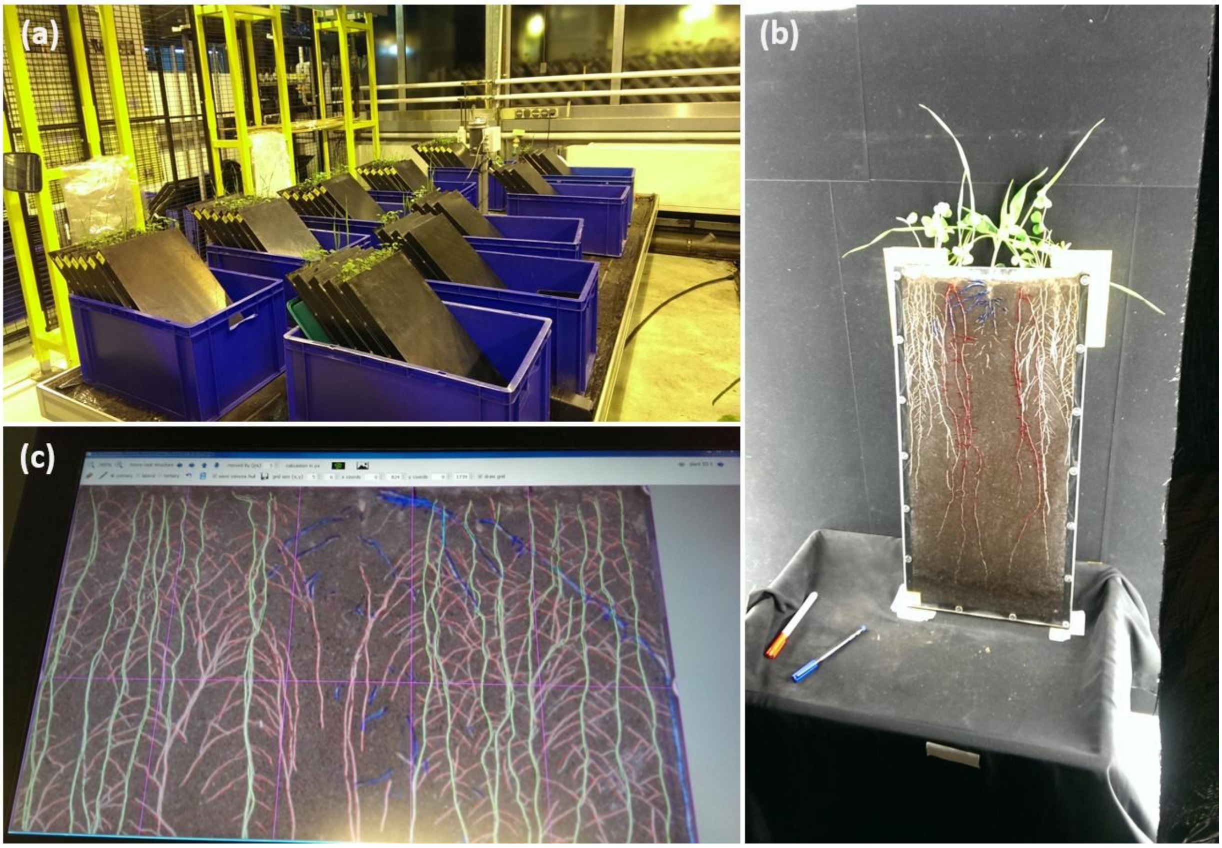


Figure 1.5. *Rhizotron experiment*: (a) rhizotrons were placed in a 45°angle to promote roots growth in the direction of the transparent interface; (b) roots drawn on the transparent interface of the rhizotron; (c) roots being digitally re-drawn in the graphic table to image acquisition.

CHAPTERS 2 TO 6 INCLUDE PUBLISHED OR SUBMITTED PAPERS. IN ORDER TO KEEP THE SAME FORMATTING FOR ALL THE CHAPTERS OF THE THESIS, THE PAPERS THAT HAVE BEEN ALREADY PUBLISHED ARE NOT SHOWN WITH THE SAME LAYOUT AS THE ONE USED IN THE FINAL PUBLICATION, BUT THEY HAVE EXACTLY THE SAME CONTENT.

PUBLISHERS AUTHORIZED THE PUBLICATION OF THE PAPERS IN THIS THESIS.

CHAPTER 2

Paper accepted in Dec 2016

The Importance of Being First: Exploring Priority and Diversity Effects in a Grassland Field Experiment

EWA Weidlich, P von Gillhaussen, BM Delory, S Blossfeld, H Poorter,
VM Temperton (2017). *Frontiers in Plant Science*, *7*, 2008.

ABSTRACT

Diversity of species and order of arrival can have strong effects on ecosystem functioning and community composition, but these two have rarely been explicitly combined in experimental setups. We measured the effects of both species diversity and order of arrival on ecosystem function and community composition in a grassland field experiment, thus combining biodiversity and assembly approaches. We studied the effect of order of arrival of three plant functional groups (PFGs: grasses, legumes and non-leguminous forbs) and of sowing low and high diversity seed mixtures (9 or 21 species) on species composition and aboveground biomass. The experiment was set up in two different soil types. Differences in PFG order of arrival affected the biomass, the number of species and community composition. As expected, we found higher aboveground biomass when sowing legumes before the other PFGs, but this effect was not continuous over time. We did not find a positive effect of sown diversity on aboveground biomass (even if it influenced species richness as expected). No interaction were found between the two studied factors. We found that sowing legumes first may be a good method for increasing productivity whilst maintaining diversity of central European grasslands, although the potential for long-lasting effects needs further study. In addition, the mechanisms behind the non-continuous priority effects we found need to be further researched, taking weather and plant-soil feedbacks into account.

INTRODUCTION

In ecology, the topics of biodiversity and ecosystem functioning (Balvanera *et al.* 2006; Isbell *et al.* 2011) and community assembly (Diamond 1975; Fukami & Nakajima 2011) are key components of the field, but have rarely being explicitly combined in experimental setups. Both the diversity of species as well as the order in which they arrive in the system can have strong effects on ecosystem functioning and community composition. As such, one might expect strong interactions between biodiversity and order of arrival. One key question is: to what extent would positive biodiversity effects found in biodiversity experiments sown at the same time be different if order of arrival was manipulated as well? Equally, within assembly experiments, what role does the diversity of the community play for establishment success? Biodiversity theory predicts that more diverse communities will be harder to invade (Elton 1958), but evidence partly supports this theory (Hector et al. 2001; Fargione & Tilman 2005) and partly does not (Stohlgren *et al.* 1999).

Since ecological communities are not static over time, understanding plant community assembly and how species can drive assembly has long been a primary goal for ecologists (Diamond 1975; Connel & Slatyer 1977). Within this context, the issue of historical contingency (dependence on history) is central, and involves the study of the effects of past events, whether biotic or abiotic (Drake 1991;

Eriksson & Eriksson 1998; Fukami 2015). This includes order of arrival of specific organisms as well as effects of disturbances (Drake 1991; Temperton et al. 2004). The study of priority effects, in contrast, focuses solely on biotic effects, and happens when organisms that first arrive at a site can significantly affect the establishment, growth, or reproduction of the species arriving later, thus influencing further assembly (Eriksson & Eriksson 1998; Fukami 2015; Vaughn & Young 2015). Priority effects can affect both the structure and functioning of ecosystems. In addition, priority effects can have a stronger influence on community composition than abiotic conditions (Fukami 2015). As such, priority effects may be a powerful tool for ecological restoration, since the order of arrival or initial plant species composition can be manipulated in ecological restoration (Schantz, Sheley & James 2015; Vaughn & Young 2015; Temperton et al. 2016). Priority effects, for example, may be useful for sending plant communities on desired trajectories for restoration.

Many experiments that test priority effects in plant communities are located in the United States, where the role of order of arrival of invasive exotic annual grasses (often from Europe) is often explored, since they can cause major species loss in native communities (Corbin & D'Antonio 2004; Martin & Wilsey 2012; Goldstein & Suding 2014; Vaughn & Young 2015). In contrast, for species-rich grasslands in Europe, the main threat for these habitats is not invasive species, but land use intensification as well as abandonment. Therefore, any incentive for farmers to keep extensively managing grasslands for diversity and higher productivity would be of benefit to species conservation in Europe (Bullock *et al.* 2007).

Biodiversity-ecosystem functioning (BEF) experiments have tested how species and functional richness affect ecosystem functioning in grasslands (Hector 1999; Roscher, Schumacher & Baade 2004), in aquatic (Callaway, Sullivan & Zedler 2003; Cardinale *et al.* 2009) and in forest systems (Bruelheide *et al.* 2014). Such experiments generally find positive diversity effects, with higher sown plant diversity leading to improved functioning of ecosystems, such as productivity, nutrient cycling. Often, positive effects found also increase over time. We know from BEF experiments in grasslands, that plant functional groups such as legumes, non- leguminous forbs, and grasses can positively affect ecosystems processes (Diaz & Cabido 2001; Pokorny *et al.* 2005). Effects of species and functional group richness as well as different combinations of functional groups can produce positive diversity effects on ecosystem functions. Legumes combined with grasses often show particularly strong diversity effects (Oelmann *et al.* 2007; Temperton *et al.* 2007; Fornara & Tilman 2008). Less is known about how relevant such BEF experiments are under natural assembly conditions (but see Bullock et al., 2007), since normally the species richness levels are maintained by weeding the plots. However, within the Jena Experiment (BEF) some studies have addressed assembly questions (e.g. Roscher et al. 2014) by stopping weeding and adding seeds and found that historical contingency was not eradicated by stopping to weed. When testing the relevance of positive biodiversity effects found in BEF experiments in a restoration context Bullock et al. (2007) sowed different seed mixtures and then allowed communities to assemble naturally. They sowed either low or high diversity seed mixtures (sowing all species at the same time) on ex-arable land, and found that sowing species-rich mixtures only once positively affected both aboveground productivity and diversity over many years.

So far experiments manipulating plant species order of arrival have mainly used controlled experimental set-ups using pots or mesocosms (Ejrnæs *et al.* 2006; Chase 2010; Moore & Franklin 2011; Stevens & Fehmi 2011; Dickson, Hopwood & Wilsey 2012; Byun *et al.* 2013; Kardol *et al.* 2013; Mason *et al.* 2013; Ulrich & Perkins 2014; Burkle & Belote 2015; Wilsey *et al.* 2015; Sikes, Hawkes & Fukami 2016). Focusing on order of arrival of different plant functional groups, Körner et al. 2008 set up an experiment with nine grassland species from three different groups (non-leguminous forbs, legumes and grasses), sowing one group before the other two. This stepwise arrival promoted different below and aboveground biomass depending on which functional group was sown first. They found priority effects of sowing legumes first, with more community biomass above and less belowground. In addition, von Gillhaussen et al. (2014) found that sowing legumes before the other functional groups affected assembly more than sowing density or sowing interval did.

With regard to field experiments, there are few studies testing order of arrival effect for more than one growing season: Collinge & Ray (2009) worked with vernal pools (wetlands), Fukami et al. (2005) manipulated initial colonization of native grasses on abandoned land, and Helsen, Hermy & Honnay (2016) tested regeneration by removing specific functional groups from grasslands. Most of the studies testing priority effects by altering order of arrival compared effects of exotic and native competition both in the field (Chadwell & Engelhardt 2008; Goldstein & Suding 2014; Young *et al.* 2014; Vaughn & Young 2015) and in controlled experiments (Grman & Suding 2010; Stevens & Fehmi 2011; Mason *et al.* 2013). These studies generally found that small differences in emergence timing can have long-lasting effects on community structure, and that initial control of exotics can increase the establishment of native perennial seedlings.

The strength of priority effects has been shown to differ depending on both soil nutrient content (Kardol *et al.* 2013), as well as on plant-soil feedback (Grman & Suding 2010; van de Voorde, van der Putten & Martijn Bezemer 2011; van der Putten et al. 2013). Kardol et al. (2013) found that effects of time of arrival depend on resource availability, and at high nutrient supply early arriving species grew quickly and reduced establishment of late arriving species.

Considering that diverse seed mixtures can improve diversity (Bullock *et al.* 2007), and that one can create priority effects by manipulating plant functional group (PFG) order of arrival (Körner *et al.*

2008), we set up a field experiment combining these biodiversity and assembly approaches. We studied the effect of order of arrival of three PFGs (grasses, legumes and non- leguminous forbs) and of sowing low and high diversity seed mixtures (9 or 21 species) on species composition and productivity on two different soil types. Our experiment is original since it combines biodiversity (sown diversity) and assembly (order of arrival) approaches, and moreover tests these factors on two different soil types. In general, we asked ourselves the question whether the effect of order of arrival is influenced by the sown diversity of the plant communities. At the same time, we wanted to know whether biodiversity effects as found in BEF experiments are influenced by order of arrival of plant functional groups, as the latter is usually not included as a factor in BEF experiments. Using our four-year field experiment we tested the following hypotheses:

1. We expect PFG order of arrival to positively affect aboveground community biomass, with higher aboveground biomass in the treatments where legumes were sown first. We expect that PFG order of arrival will not affect the number of species but rather the functional composition of the community. More specifically, we expect that the PFG sown first will dominate each treatment (i.e. causing a priority effect).
2. Sowing high diversity seed mixtures (sown diversity) will positively affect community aboveground biomass and number of species managing to establish.
3. We expect an interaction effect between the order of arrival and sown diversity treatments. In particular, we hypothesize that the highest aboveground biomass will be found in the high diversity treatment where legumes were sown first.
4. We expect that the outcome of PFG order of arrival and sown diversity will be modulated by soil type.

MATERIAL AND METHODS

Experimental site

The Priority Effect Experiment is located on an ex-arable field southeast of Jülich (Germany -altitude 94 m - 50°53’51.53” N, 6°25’21.09” E). Mean annual air temperature is 9.9° C and mean annual precipitation is 699 mm. The site was cultivated as an arable field until 2006 (mainly for the cultivation of vegetables and root crops) and was then used as extensive grassland (with typical grassland species sown by the farmer) from 2006 until the establishment of the experiment in 2012. Prior to the experiment the field was ploughed and raked multiple times during the winter 2011/2012 to counteract germination of weeds from the soil seed-bank and to create bare ground.

Four soil profiles were dug out in 2011 at the field site, and as result of it, the experiment was set up on two areas (A and B - Fig. 2.1) reflecting the soil types Stagnic Cambisol (normally productive soil type) in area A and Anthrosol (modified by human activity) in area B (slightly elevated – approximately 1.8 m higher than A). The soil survey followed the official German soil mapping guidelines (Sponagel 2005).

Figure 2.1. Aerial image showing the experimental design used on the two sites (area A and area B). Each plot is described by a code containing the following information: the sown diversity (HD, high diversity; LD, low diversity), the PFG order of arrival (F, forbs-first; G, grasses-first; L, legumes-first; and C, controls where all PFGs were sown at the same time) and the replicate number (n= 4 per area). The plots without any legend were the blank ones where nothing was sown (data not shown).

Experimental design and species selection

The main treatments of the experiment were the sown diversity (2 levels: high or low diversity), the PFG order of arrival (4 levels: grasses, forbs or legumes sown first and all PFGs sown at the same time) and the soil type (2 levels: area A and area B). Areas A and B had exactly the same treatment factors and four replicates (n= 4 per soil type) resulting in a total of 64 plots of 4 m x 4 m (Fig. 2.1).

Two different sown diversity levels were used in the experiment to assess the effects of species richness on ecosystem functioning and diversity outcomes in the assembling communities. In total, a fixed set of 21 common species (7 forbs, 7 grasses and 7 legumes) was selected for the high diversity communities. A randomly chosen, fixed subset of 9 species (3 forbs, 3 grasses and 3 legumes) was selected to represent low diversity communities (Table 2.1). When choosing species, we aimed to reflect those which are relatively common and dominant in grasslands of the area. The target plant community is a semi-natural species-rich mesotrophic grassland, consisting of typical central European grassland species (Ellenberg 1988). Species were selected taking their performance in previous controlled experiments (von Gillhaussen *et al.* 2014) and pre-experiments into account,. Species were classified into three different PFGs: forbs (non-leguminous), grasses and legumes. These species categories were intentionally kept broad, to create general functional envelopes which each include plant species that differ significantly in their functional and morphological traits (based on Roscher, Schumacher & Baade 2004). Forbs included any non-leguminous, non-grass species. Grasses included members of the Poaceae family, and these species are morphologically most different from the other groups. Legumes are forbs of the Fabaceae family which differ from species of other PFGs in their ability to fix atmospheric N_2.

The PFG order of arrival treatment was created by sowing the species of one PFG on April 19th 2012 (or all PFGs at the same time in the control plots), while the species from the other PFGs were sown on May 31st 2012, resulting in four treatment levels: forbs-first (F-first), grasses-first (G-first), legumes-first (L-first) and control. The length of the interval between sowing events was based on a previous greenhouse study (von Gillhaussen *et al.* 2014), where a 6-week interval produced larger priority effects than a 3-week interval. Before the 2nd sowing all plots were mown, to allow subsequently sown species a better chance to germinate and establish, and to increase complementarity between PFGs. None of the plots was weeded thus allowing colonization and natural assembly processes to occur after the sowing events.

In each plot, the sowing density was 5 g/m2 divided equally among the species of each mixture. The seed mixtures were mixed with sand to improve handling and ensure an even distribution on the plots at the time of sowing. The number of seeds taken for each species was adjusted according to their thousand seed weight. Seeds were sown by hand into the plots, and afterwards each plot was flattened to ensure proper adherence of seeds to soil particles and to avoid granivory. A non-clonal grass species, *Festuca rubra spp. commutatis*, was sown in the areas between the plots as lawn paths.

Table 2.1. Plant species chosen for the Priority Effect Experiment with the respective PFG assigned for each species. Species were selected from a species pool of the typical central European grassland types. Species pools for high and low diversity (HD and LD) mixtures were fixed (not random). Presence of species in a sown diversity is denoted by an “x”.

Plant functional groups (PFG)	**Species**	**Code in PCA**	**Sown diversity:**	
			high	**low**
Forbs	*Achillea millefolium*	F1	x	x
	Crepis biennis	F2	x	
	Galium verum	F3	x	
	Geranium pratense	F4	x	
	Leontodon hispidus	F5	x	
	Leucanthemum vulgare	F6	x	x
	Plantago lanceolata	F7	x	x
Grasses	*Arrhenatherum elatius*	G1	x	
	Bromus erectus	G2	x	
	Dactylis glomerata	G3	x	x
	Festuca pratensis	G4	x	X
	Helictotrichon pratense	G5	x	
	Holcus lanatus	G6	x	x
	Poa pratensis	G7	x	
Legumes	*Lathyrus pratensis*	L1	x	
	Lotus corniculatus	L2	x	x
	Medicago sativa	L3	x	x
	Onobrychis vicifolia	L4	x	
	Trifolium hybridum	L5	x	
	Trifolium pratense	L6	x	x
	Trifolium repens	L7	x	

Sampling and data collection

To assess the effects of our treatments on community composition, we estimated the plant cover of each species prior to the harvest of aboveground biomass using the Braun-Blanquet method modified by Londo (1976). We assessed the cover of non-target (mainly weedy) species as a total cover for this group, but this data is not shown in the graphs. Since the non-target species were not identified down to species level and hence could not be assigned to different PFG, we decided to exclude them from the analyses. Even though the weed cover in the first year was up to 20%, this reduced drastically due to mowing over the years (less than 1% in 2015).

Total aboveground biomass (dry matter yield, g/m2) was measured in June (2012, 2013, 2014 and 2015) and September (2012, 2013 and 2014). Here we only report the peak biomass data from June of each year. Two randomly positioned 0.1 m2 rectangles (20 × 50 cm) were harvested from each plot at each harvest. All aboveground plant material within the rectangle was cut 2 cm above the soil surface and samples were dried at 70°C (until constant weight) before weighing. During the harvest of 2014, harvested plant material was sorted into species, in order to have biomass data per species. All plots were mown twice per growing season (according to agricultural practice in managed mesotrophic grasslands), in July and September, except in 2015 when we harvested only once at peak biomass in June.

Total carbon (C), nitrogen (N), potassium (K) and phosphorus (P) in soil samples were measured in April 2012 and in September 2014 by pooling three soil cores (each 40 cm x 5 cm) into one sample per plot, giving a total of 64 soil samples per element. Soil samples collected from each plot of the experiment were analysed for % C, %N, %K and %P (VarioelCube Elementar and ICP-OES methods). For %P in soil, we were only able to analyse the samples from 2014, since in 2012 the measurements were below the detection limit of the method.

Statistical analyses

Our field experiment was performed over four years and is multi-factorial in design, with PFG order of arrival and sown diversity as the main fixed factors. Because we were interested to see if the effects of PFG order of arrival and sown diversity on the measured variables changed between experimental sites, we also considered soil type as a fixed factor. All statistical analyses were performed using R 3.3.1 (R Core Team 2016) and an alpha value of 5%.

The effects of treatments on aboveground biomass and on species richness were analysed with linear mixed effects models following the procedure described by Zuur et al. (2009). For each variable, we started by fitting a model containing all explanatory variables and all possible interactions between PFG order of arrival, sown diversity and soil type. First, we found the optimal structure of the random component of each model using restricted maximum likelihood (REML) estimators. Using year as a random factor, we compared two different random structures: (1) no random term (using the generalized least squares method) and (2) a random intercept model. The model with the lowest Akaike Information Criterion (AIC) value was then selected. For both aboveground biomass and species richness, a random intercept model was retained for further statistical analyses. We then found the optimal fixed structure of each model by dropping the non-significant terms (based on F-statistics). The linear mixed models were fitted with the lme function of the nlme package (Pinheiro *et al.* 2016).

Because we were interested to see if each of the factors tested in our grassland experiment had an effect on aboveground biomass and species richness on each soil type in each year, the data were also analysed using one-way ANOVAs, where either PFG order of arrival or sown diversity were the fixed factor. When the null hypothesis was rejected, the mean treatment values were compared with a Newman and Keuls test performed with the R package agricolae (de Mendiburu 2015).

The influence of PFG order of arrival and sown diversity on species composition over the years was analysed using a principal component analysis (PCA). A single PCA was performed on a dataset containing the cover data of 21 plant species (2012, 2013 and 2015) using the R package FactoMineR (Husson *et al.* 2016). The PCA was performed using a correlation matrix constructed from scaled variables. In this paper, we considered that a variable (plant species) contributed significantly to a principal component (PC) if its contribution (expressed in %) was greater than the contribution that would have been observed if all variables contributed equally to a component. In our case, this threshold value was equal to 4.8% and was calculated as 100 divided by the number of species for which cover data were available (21).

We analysed the soil chemistry data using linear models, because we were interested to see the effects of our experimental factors (PFG order of arrival, sown diversity) as well as soil type and year. For each of the four soil variables (C, N, P, K), we started by fitting a model containing all explanatory variables and all possible interactions between the factors. Then, we simplified the model by dropping the non-significant terms based on F-statistics. The linear models were fitted using the lm function of R.

RESULTS

Effects of PFG order of arrival and sown diversity on aboveground biomass

The mixed effect model run over the whole four-year dataset showed that the aboveground biomass was significantly affected by PFG order of arrival ($P=0.0011$), but not by sown diversity. We did not find any significant interaction between PFG order of arrival, sown diversity and soil type, therefore, the graphs are shown separately for each factor (without interaction). Since we were explicitly interested in how sown diversity and order of arrival affected the biomass over time, we also analysed the effects of PFG order arrival (Fig. 2.2) and sown diversity (Fig. 2.3) on aboveground biomass separately for each year and each soil type. The mean biomass over the four years was 656 g/m^2 in area A and 731 g/m^2 in area B.

Our results from 2012 showed that the highest biomass values were obtained when all PFGs were sown at the same time, and when forbs (area A) or legumes (areas A and B) were sown first (Fig. 2.2). In the second year of the experiment (2013), there were no significant differences in biomass between order of arrival treatments (Fig. 2.2). In 2014, we found that legumes promoted priority effects only in area B, where the legumes-first treatment had significantly higher biomass than the other treatments (Fig. 2.2). The same pattern was found in 2015, but this time without any statistically significant differences (Fig. 2.2).

Looking at the effect of sown diversity, we found that the low diversity plots had higher biomass in 2013, while no significant differences were found in the other years (Fig. 2.3).

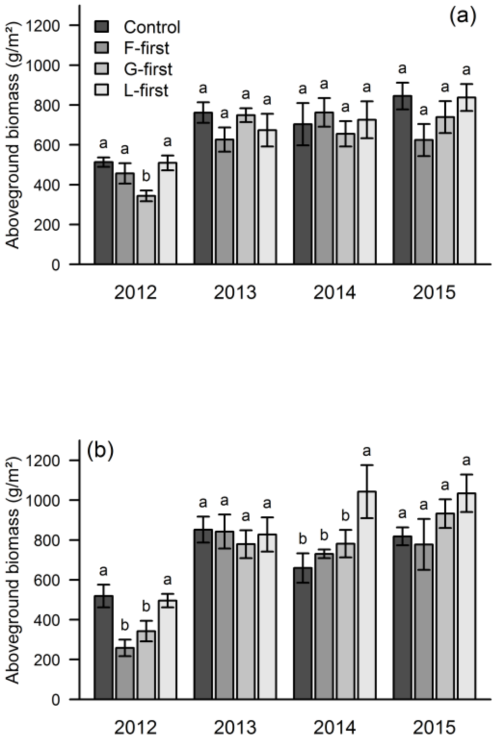


Figure 2.2. Influence of PFG order of arrival on aboveground productivity over four years of a grassland experiment. The results are shown separately for the two experimental sites: area A (A) and area B (B). In control plots, all PFG were sown at the same time. In the other plots, the PFG order of arrival was experimentally manipulated (F-first, forbs sown first; G-first, grasses sown first; L-first, legumes sown first). The values are means plus/minus one standard error of the mean (n= 4). Within each year, different letters show significant differences between treatments (one-way ANOVA followed by a Newman and Keuls test, $P < 0.05$).

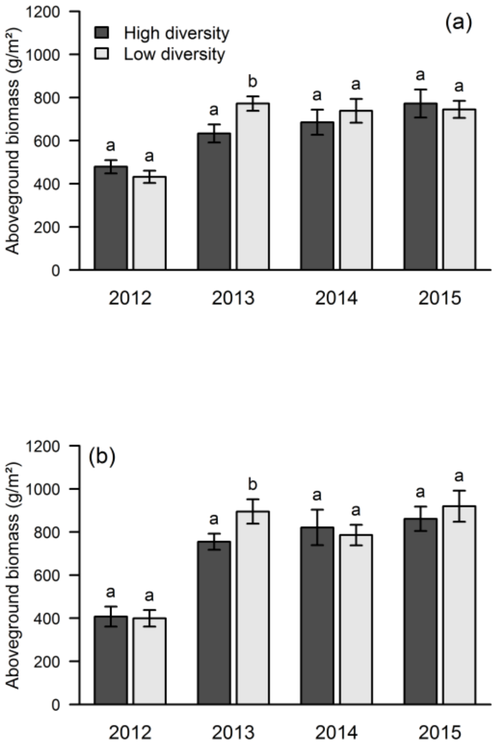


Figure 2.3. Influence of sown diversity on aboveground productivity over four years of a grassland experiment. The results are shown separately for the two experimental sites: area A (A) and area B (B). A total of 21 and 9 species were sown at the beginning of the experiment in high and low diversity plots, respectively. The values are means plus/minus one standard error of the mean (n= 4). Within each year, different letters show significant differences between treatments (one-way ANOVA followed by a Newman and Keuls test, P < 0.05).

Effects of PFG order of arrival and sown diversity on number of species

The results of the mixed effects model showed that the number of species that managed to establish themselves was affected by PFG order of arrival (P=0.0004) and sown diversity (P<0.0001). We also found a significant interaction between sown diversity and soil type (P= 0.015). No other significant interaction was observed. The mean number of species in area A was slightly higher than in area B (7.7 and 6.5 species per plot, respectively).

Our results showed that PFG order of arrival significantly affected the number of species in the first (area A) and second year (areas A and B) of the experiment because the control treatment had a higher number of species in comparison with the other treatments (Fig. 2.4). In 2014, we did not find any statistical difference between the treatments in both areas with regard to the species richness. In the following year (2015), we found significantly more species in the F-first plots of area A in comparison with the other plots of the same area, but in area B, the species richness was not affected by PFG order of arrival.

With regard to the effect of sown diversity, the number of species that managed to establish was higher in the high diversity treatment in areas A and B in 2012, while only area A showed this pattern in 2013 and 2014 (Fig. 2.5). In 2015, we found more species in the high diversity plots only in area B (Fig. 2.5).

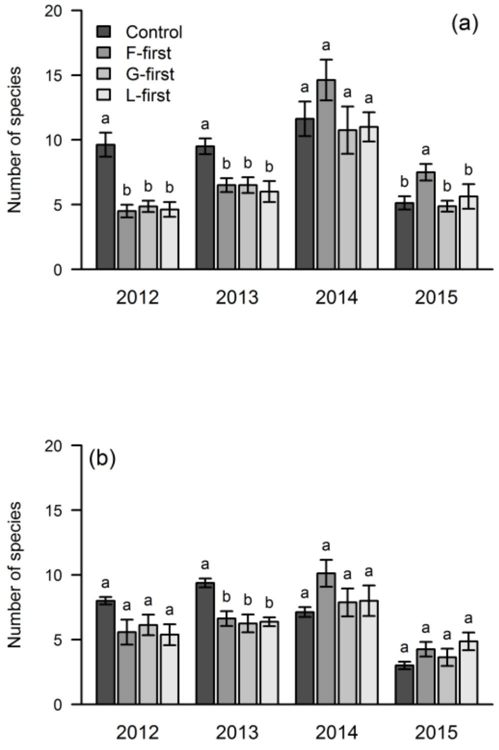


Figure 2.4. Influence of PFG order of arrival on species richness over four years of a grassland experiment. The results are shown separately for the two experimental sites: area A (A) and area B (B). In control plots, all PFG were sown at the same time. In the other plots, the PFG order of arrival was experimentally manipulated (F-first, forbs sown first; G-first, grasses sown first; L-first, legumes sown first). The values are means plus/minus one standard error of the mean (n= 4). Within each year, different letters show significant differences between treatments (one-way ANOVA followed by a Newman and Keuls test, $P < 0.05$).

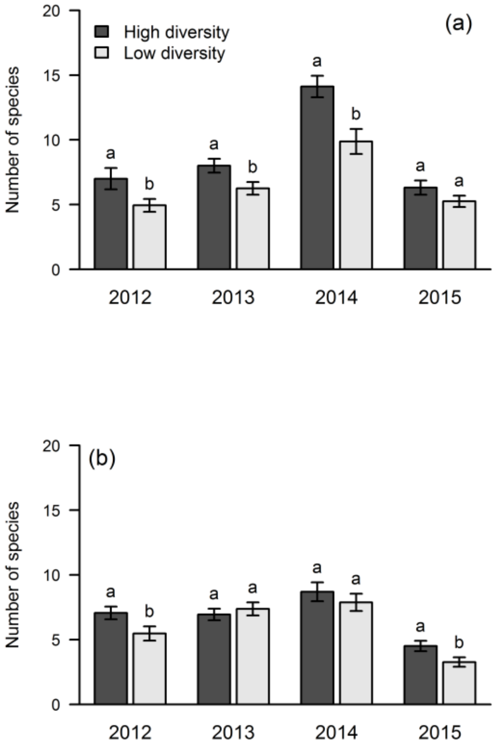


Figure 2.5. Influence of sown diversity on species richness over four years of a grassland experiment. The results are shown separately for the two experimental sites: area A (A) and area B (B). A total of 21 and 9 species were sown at the beginning of the experiment in high and low diversity plots, respectively. The values are means plus/minus one standard error of the mean (n= 4). Within each year, different letters show significant differences between treatments (one-way ANOVA followed by a Newman and Keuls test, P < 0.05).

Effects of PFG order of arrival and sown diversity on community composition

The PCA showed that in the first (2012) and second (2013) year of the experiment, the F-first, G-first and L-first plots mainly consisted of the plant species belonging to the PFG sown first, as expected. In 2012, the species composition of the control plots differed between the two sown diversity levels. When the control plots were sown in low diversity sowing (positive PC2 values), they had on average a greater forb cover than the control plots sown in the high diversity sowing, which were composed mainly by legumes (negative PC2 values). In contrast to the first two years however, from 2013 to 2015, the species composition of the plots in all treatments converged to a state dominated by two grasses (*Holcus lanatus* and *Dactylis glomerata*) regardless of the PFG order of arrival and sown diversity treatments.

More specifically, the two first principal components accounted for 31% of the total variance. The species *Holcus lanatus* (18.1%), *Trifolium pratense* (13.6%), *Lotus corniculatus* (11.1%), *Trifolium* *repens* (7.5%), *Trifolium hybridum* (7.2%), *Achillea millefolium* (6.5%), *Medicago sativa* (6.3%), *Onobrychis vicifolia* (5.7%) and *Dactylis glomerata* (5.5%) contributed significantly to PC1 (Fig. 2.6a,b). Essentially, PC1 can be interpreted as an axis separating plots dominated by *Holcus lanatus* and *Dactylis glomerata* from plots dominated by forbs and legumes (Fig. 2.6). With regard to PC2, the plant species that had the greatest contributions were *L. vulgare* (18.4%), *Achillea millefolium* (15.4%), *Plantago lanceolata* (11.6%), *Galium verum* (9.2%), *Trifolium hybridum* (8.4%), *Onobrychis vicifolia* (8.0%) and *Crepis biennis* (7.9%), and mainly separated plots dominated by forbs from the ones dominated by legumes.

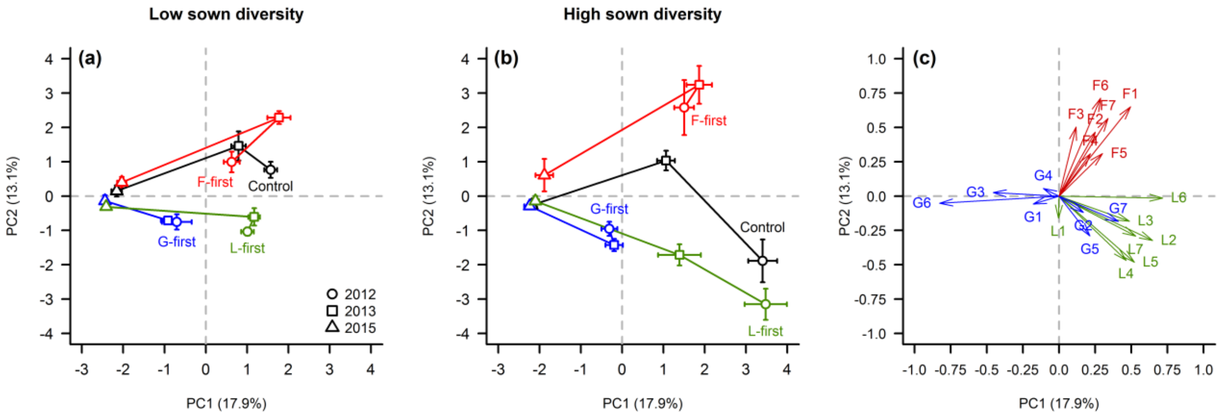


Figure 2.6. Influence of PFG order of arrival on species composition. Cover data were analyzed with a PCA. In panels (A) and (B), a score plot constructed with the two first principal components (PC) is shown for each sown diversity level. The panel (C) shows the correlation coefficient of each plant species with PC1 and PC2. Horizontal and vertical error bars are standard errors according to PC1 and PC2, respectively. In panel (C), we used a code made of one letter (refers to the PFG; F=forbs, G=grasses, L=legumes) and one number for naming the plant species. A full description of the code can be found in table 1.

Soil chemistry

Our experimental factors (sown diversity and PFG order of arrival) had no significant effect on the soil parameters analysed in both years. Data analyses for soil C, N and K showed a highly significant interaction between year and soil type (C: $P<0.0001$; N: $P<0.0001$; K: $P=0.0009$).

Soil samples from area B contained significantly more C and N in 2014, whereas in 2012, these parameters did not differ between the two experimental sites. Soil K in area A was higher than in area B only in 2012, while the measured values did not differ between areas in 2014. We observed a strong decrease of soil K from 2012 to 2014 (Supporting information 2.1).

DISCUSSION

We found a transient significant effect of PFG order of arrival over the four years on aboveground biomass, number of species and species composition. We could partially confirm our first hypothesis that PFG order of arrival would affect biomass and species composition, but could not confirm an effect on the number of species. The effects of order of arrival found on biomass and species composition were only partially confirmed since they changed over time. In the first year, aboveground biomass was significantly higher in L-first and in control plots than in G-first and F-first plots. This effect was probably related to these treatments having similar species compositions. In the second year this pattern disappeared, while in the third year we found significantly more aboveground community biomass in legume-first plots, but only in area B. Our results suggest that legume order of arrival was partly driving aboveground biomass, but that this legume effect was not continuous over time, such that we did not find a clear continuous priority effect. Our field experiment results are nevertheless consistent with outcomes in similar controlled experiments (Körner *et al.* 2008; von Gillhaussen *et al.* 2014), in that legumes played an important role in our field experiment, albeit not as clearly as found under controlled conditions.

The two main processes behind priority effects are niche pre-emption and niche modification (Fukami 2015). In the first mechanism, species that arrive first have the advantage of early establishment, and hence tend to perform better due to asymmetric competition. In the second mechanism, early-arriving species directly modify the abiotic environment, thereby changing the type of niches that are available, thus affecting establishment opportunities for later arriving species that colonize the community. Legumes grow fast aboveground and forbs generally invest proportionally less in root biomass than grasses as seen in Körner et al. (2008) and Poorter et al. (2015), and found in 2012 and 2014 in our experiment (data not shown here). A possible mechanism for the sporadic legume priority effect we found in the field, is that the PFG arriving after legumes had more opportunities for root and nutrient foraging due to the smaller root systems of the legumes that arrived first (a process known as N sparing; Felten et al. 2016). Since (1) the species composition of control and legumes-first plots was very similar in the first year (dominated by legumes) and (2) we found a significant legumes-first priority effect in the third year, probably the N sparing mechanism could have played a role in our system. For some reason however, this effect was not consistent over time. It is also possible that later neighbours of legumes benefitted from N transfer, where the neighbours of N2-fixers profit from legume-fixed N via direct or indirect transfer (sensu Temperton et al. 2007; Bessler et al. 2012).

The number of species found in plots in 2012 and 2013 show strong priority effects of sowing a PFG before another, since the number of species establishing was higher when all PFGs were sown at the same time. However, in the third year the species richness was no longer different between the treatments, while in 2015 more species were found in the F-first plots (although only in area A). These results partially agree with our first hypothesis, that PFG order of arrival would not affect the number of species as much as the species composition. With regard to the latter, we expected that the PFG sown first would dominate each treatment, possibly causing a priority effect, and our results showed this in the first and second year. The F-first, G-first and L-first plots were mainly covered by the plant species belonging to the PFG sown first, while in control plots, the legumes were characterized by a slightly higher cover than the average. On the other hand, the following years showed that the plots had higher cover of grasses (mainly *Holcus lanatus* and *Dactylis glomerata*). It seems that the community composition was possibly tracking natural grassland succession in the experiment, where legumes are often more common in the beginning, with grasses gradually taking over.

Our results agree with Ejrnæs et al. (2006) who found that species richness and invasibility is controlled by environment but that species composition is determined by plant order of arrival. This emphasizes that historical contingency can significantly change the outcome of community assembly. In contrast, another study concluded that historical effects may be lost within a decade, and found significant but transient effects of seeding, order of colonization, and frequency of colonization on species abundance (Collinge & Ray 2009). Experiments testing a two-week planting advantage between exotic and native plant species (in field conditions) also found that although priority effects reduced over time, small differences in emergence timing had long-lasting effects in the community (Vaughn & Young 2015).

Our second hypothesis that sown diversity would promote higher aboveground biomass and number of species was confirmed for the effect on number of species but not for biomass. As expected, sown diversity clearly affected the number of species that established. The high diversity treatments usually had a higher number of species but we did not find a significant effect of sown diversity on biomass. This is in contrast to most of the BEF experiments, which normally find an effect of sown diversity on aboveground biomass and other ecosystem processes (Balvanera *et al.* 2006; Marquard *et al.* 2009).

The lack of significant effects of sowing high or low diversity seed mixtures on biomass in 2012, 2014, and 2015 might be explained by the fact that our low diversity treatment consisted of rather dominant species within the three PFGs. Thus, these dominant species could also perform very well in the low diversity treatment, and were more competitive than the additional species in the high diversity (21 species) treatment. In the Bullock et al. (2007, 2001) studies that found higher aboveground biomass when sowing more diverse seed mixtures (as we expected to find), most of the low diversity treatments did not include legume species, while all the high diversity treatments did. This may explain why they found more sustained effects of sown diversity over time than was found in our study (which had legumes in both treatments).

In our third hypothesis, we expected that L-first plots sown with high diversity seed mixtures would have the highest biomass. But since there was no significant interaction between PFG order of arrival and sown diversity, this hypothesis was not confirmed by our results. As stated above, it is possible that the species present in the low diversity plots tended to be quite dominant over time, thus hindering the effect of sown diversity and its interaction with order of arrival. On the other hand, we know from other BEF experiments that the species driving positive biodiversity effects are often the dominant ones, the identities of which change each year (Allan et al. 2011).

The lack of experiments combining biodiversity and assembly effects (sown diversity and PFG order of arrival) emphasizes the need for more studies to test this interaction over a range of different soil types and habitats, as well as taking species dominance explicitly into account.

Since we found a significant interaction between soil type and sown diversity, but did not find such interaction between soil type and PFG order of arrival, we could only partially confirm our fourth hypothesis that differences in soil type would affect the outcome of priority effects. Overall, irrespective of experimental treatments, area A with less fertile soil had a lower biomass but a higher number of species, while area B with more fertile soil had a higher biomass and lower number of species. Soil K decreased significantly between 2012 and 2014 but this was not affected by the experimental factors. Since K is essential for N2-fixation in legumes (PDA-Potash Development Association 2015), this may have partly driven the overall change in dominance from legumes to grasses (irrespective of experimental treatments). This outcome of our experiment is thus not a desirable outcome for grassland restoration, where the goal is to develop a predominance of forbs mixed in with grasses, as is commonly found in central European grasslands (Kirmer *et al.* 2012). We now therefore suggest further research into the reason and effects of the reduction in soil K. Even though we could not find significant interactions between soil type and PFG order of arrival, priority effects were more evident in area B. Since area B had higher concentrations of soil C and N than area A in 2014, plants in this area probably had enough nutrients and invested more in shoot growth than in roots thus promoting higher aboveground biomass. Similar results showing that plant species establishment depends on resource availability were found in a mesocosm experiment, where at high nutrient supply early arriving species grew quickly and reduced establishment of late arriving species (Kardol *et al.* 2013).

Ideally, to be able to use biodiversity and order of arrival effects as tools for economically viable ecological restoration in cultural landscapes, the legumes-first treatment would lead to higher community biomass at least in some years. In addition, functional group composition would ideally be more balanced than in the other treatment levels, but species number would not be affected by it (based on the greenhouse experiment of von Gillhaussen et al. 2014). Even though we only partly found clear priority effects in our experiment, the results may have implications for restoration. For instance, if the non-continuous priority effects we found were more general in nature, this would mean that using such priority effects to steer communities along a desired trajectory would not be a viable procedure for restoration management. Thus, further experiments are now needed to be able to clarify whether manipulating order of arrival may be useful in a restoration context or not, using a range of different soil types and grassland habitats.

Our research suggests that sowing legumes first may be a valuable tool in creating more productive yet diverse communities in central European grasslands, but this may be context-dependent. The potential for long-lasting effects needs further study in different soil types and with different grassland types (e.g. oligotrophic vs mesotrophic). In addition, understanding the mechanisms behind the non-continuous priority effects we found needs to be tested using a range of different experiments that address issues of weather and plant soil feedback (van de Voorde *et al.* 2011).

SUPPORTING INFORMATION CHAPTER 2

Supporting information 2.1. Mean percentage of soil nitrogen (N), phosphorus (P), potassium (K) and carbon (C) in the sown diversity and PFG order of arrival treatments, separated by year (2012, 2014) and areas A and B. Standard deviation is indicated by ±.

Sown diversity	**PFG order of arrival**	**Soil chemistry**						
		2012			**2014**			
		% C	**% N**	**% K**	**% C**	**% N**	**% K**	**% P**
		AREA A						
High diversity	Control	0.969 ±0.02	0.094 ±0.04	1.633 ±0.00	1.059 ±0.09	0.106 ±0.01	0.067 ±0.01	1.489 ±0.06
	F-first	0.931 ±0.05	0.093 ±0.05	1.578 ±0.00	1.004 ±0.17	0.102 ±0.01	0.068 ±0.00	1.573 ±0.04
	G-first	0.935 ±0.04	0.093 ±0.04	1.598 ±0.00	0.898 ±0.05	0.092 ±0.00	0.084 ±0.00	1.555 ±0.08
	L-first	0.979 ±0.06	0.096 ±0.02	1.665 ±0.00	0.983 ±0.11	0.105 ±0.01	0.070 ±0.01	1.633 ±0.01
Low diversity	Control	0.966 ±0.04	0.094 ±0.06	1.595 ±0.00	1.030 ±0.03	0.109 ±0.00	0.069 ±0.02	1.518 ±0.04
	F-first	0.961 ±0.06	0.094 ±0.07	1.528 ±0.00	1.015 ±0.06	0.100 ±0.00	0.088 ±0.01	1.623 ±0.05
	G-first	0.972 ±0.03	0.095 ±0.06	1.690 ±0.06	0.953 ±0.10	0.097 ±0.01	0.079 ±0.01	1.543 ±0.09
	L-first	0.971 ±0.02	0.096 ±0.02	1.645 ±0.00	0.949 ±0.19	0.107 ±0.01	0.072 ±0.01	1.595 ±0.05
		AREA B						
High diversity	Control	0.949 ±0.03	0.098 ±0.15	1.568 ±0.00	1.218 ±0.08	0.128 ±0.01	0.079 ±0.00	1.548 ±0.04
	F-first	0.964 ±0.04	0.098 ±0.09	1.575 ±0.01	1.114 ±0.07	0.122 ±0.01	0.071 ±0.00	1.550 ±0.04
	G-first	1.022 ±0.07	0.102 ±0.08	1.608 ±0.00	1.064 ±0.12	0.115 ±0.01	0.067 ±0.02	1.485 ±0.15
	L-first	0.960 ±0.09	0.098 ±0.09	1.548 ±0.00	1.226 ±0.12	0.133 ±0.01	0.076 ±0.01	1.569 ±0.08
Low diversity	Control	0.950 ±0.02	0.099 ±0.04	1.580 ±0.00	1.169 ±0.08	0.135 ±0.01	0.068 ±0.02	1.575 ±0.04
	F-first	0.961 ±0.04	0.095 ±0.16	1.485 ±0.00	1.214 ±0.21	0.133 ±0.02	0.074 ±0.01	1.615 ±0.04
	G-first	0.971 ±0.06	0.100 ±0.08	1.480 ±0.01	1.152 ±0.08	0.126 ±0.01	0.080 ±0.00	1.625 ±0.05
	L-first	0.931 ±0.05	0.095 ±0.07	1.465 ±0.00	1.141 ±0.04	0.128 ±0.00	0.075 ±0.01	1.600 ±0.00

CHAPTER 3

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Priority effects caused by plant order of arrival affects belowground productivity

EWA Weidlich, P von Gillhaussen, JFJ Max, BM Delory, ND Jablonowski, U Rascher, VM Temperton. Journal of Ecology (in press).

ABSTRACT

1. Plant species that arrive first in the system can affect assembly (priority effects). However, effects of order of arrival of different plant functional groups on root development have not yet been investigated under field conditions.
2. We measured standing and fine root length density in the first and third year of a grassland field experiment. We wanted to know if manipulating plant functional group order of arrival would affect root development, and if priority effects are modulated by soil type.
3. Sowing legumes first created a priority effect that was found in the first and third year, with a lower standing root length density in this treatment, even though the plant community composition was different in each of the studied years. Fine root length density was not affected by order of arrival, but changed according to the soil type.
4. *Synthesis:* We found strong evidence that sowing legumes first created a priority effect belowground that was found in the first and third year of the field experiment, even though the functional group dominance was different in each of the studied years.

INTRODUCTION

Plants species that first arrive at a site can significantly affect the establishment, growth, or reproduction of the species arriving later, thus influencing further assembly (Eriksson & Eriksson 1998; Fukami 2015). This so called priority effect, can lead to alternative states in vegetation resulting in a range of different community trajectories (Temperton V.M. 2004; Martin & Wilsey 2012). Thus, the species that arrive and establish first at a site (and their traits) can significantly affect ecosystem structure, as well as ecosystem functioning and services. Priority effects caused by order of arrival can even have stronger influence on community composition than abiotic conditions (Fukami 2015). As such, understanding when priority effects most occur, how strong and persistent they are over time should improve our knowledge of community assembly and could be relevant to ecological restoration. Since plants do not arrive at the same time at a site, priority effects form an important aspect of community assembly, whereby the species that arrive first can significantly affect the system. Thus, understanding better what regulates assembly can bring us useful information on how to return a degraded area to a functioning ecosystem.

Most of the experiments manipulating plant order of arrival normally test the effects of this stepwise arrival on aboveground parameters such as shoot biomass (Ladd & Facelli 2008; Grman & Suding 2010; Dickson *et al.* 2012; Kardol *et al.* 2013; Goldstein & Suding 2014), number of species (Ejrnæs *et al.* 2006; Grman, Bassett & Brudvig 2013; Helsen *et al.* 2016), or abundance (Daehler & Goergen 2005; Antonio *et al.* 2014; Schantz *et al.* 2015). In contrast, only few studies have tested the effects of order of arrival on belowground development, and all of these experiments are under controlled conditions. In addition, these experiments assessed the role of assembly history and performance of native versus exotic (Orloff *et al.* 2013; Sikes *et al.* 2016) with few experiments testing interactions purely between native species (Körner *et al.* 2008; Moore & Franklin 2012). To our knowledge, the effect of order of arrival on belowground development has not yet being tested in the field. Thus, we lack field knowledge on how assembly history drives root functioning and development. Studies reporting belowground data from field experiments are demanding and time-consuming, but necessary to evaluate whether order of arrival can affect root development in the same way it has been reported to affect aboveground tissues. In addition, understanding what happens belowground can help us to answer questions that cannot be solved when only looking the aboveground outcome, especially since we know that belowground development can lag behind the aboveground, for example, during biodiversity experiments (Ravenek *et al.* 2014). This will also help in guiding plant management in agriculture or restoration settings (Palmer, Ambrose & LeRoy Pff 1997).

Previous work, testing the role of assembly history and success of natives and exotics in controlled experiments, found that initial size of competing plants can have long lasting effects on the plant community above and belowground performance and can inhibit the later species establishment (Orloff *et al.* 2013). Sikes *et al.* (2016) also found that small differences in early arrival history, during the first two weeks of assembly, were sufficient to affect plant above and belowground performance for six months. Studying the effects of order of arrival among native species, Moore & Franklin (2012) found in controlled experiments that the root biomass of later species were reduced after flooding and drought compared to species that arrived first, while Körner *et al.* (2008) showed that sowing different functional groups at different time led to higher aboveground and lower belowground biomass when legumes were sown first. Körner *et al.* (2008) hypothesized that lower belowground productivity in treatments where legumes were sown first was due to smaller legume root systems and hence increased nutrient availability for non-legume neighbours arriving at a later time (the so-called N sparing effect, see Temperton *et al.* 2007). Few studies tested the potential role of different plant functional groups arriving first such as the study by Körner *et al.* (2008), despite a vast amount of research in biodiversity-ecosystem functioning experiments showing the essential driving role of combining specific plant functional groups (particularly legumes and grasses).

Since we know that plant performance aboveground can be affected by altering the order of arrival of different plants or plant functional groups (Goldstein & Suding 2014; Cleland *et al.* 2015; Vaughn & Young 2015), in 2012 we set up a field experiment testing the effects of order of arrival of three plant functional groups (grasses, legumes and non- leguminous forbs) as well as high versus low diversity seed mixture on assembly (Weidlich *et al.* 2017). We found that the order of arrival of different plant functional groups affected the aboveground biomass and community composition, but these effect were not continuous over time. In this present study, however, we wanted to test if this stepwise arrival would also affect root development as we partially found aboveground. Thus, we measured standing and fine root development in the first and third year (2012 and 2014) in a sub-set of plots of our field experiment. More specifically, we wanted to know (1) whether priority effects caused by manipulating plant functional groups order of arrival would also affect belowground development, and (2) if these priority effects were modulated by soil type.

MATERIALS AND METHODS

The priority effect experiment

We measured root length in the Priority Effect experiment (Jülich, Germany), a grassland field experiment, which was set up in 2012. The overall aim was to combine assembly (order of arrival) and biodiversity (sown diversity) approaches. The main factors tested in this experiment were the effects of order of arrival (4 levels: grasses, forbs or legumes sown first and all plant functional groups sown at the same time), of soil type (2 levels: area A and area

B), and of sowing low and high diversity seed mixtures (2 levels: 9 or 21 species) on species composition and biomass. Area A and B had the same treatments and four replicates each resulting in a total of 64 plots of 4 m x 4 m (more detailed information in Weidlich *et al.* 2017). Results of aboveground biomass and species composition were reported earlier in Weidlich *et al.* (2017), and in this present study we show the results of belowground development, collected in a sub-set of plots (three order of arrival levels and low diversity plots) in the first and third year of the experiment (2012 and 2014).

The order of arrival treatment was created by sowing the species of one PFG (or all PFGs at the same time in the control plots) with a six weeks sowing interval before the other PFGs, resulting in four treatment levels: forbs-first (F-first), grasses-first (G-first), legumes-first (L-first) and control. Before the second sowing, all plots were mown, to allow subsequently sown species a better chance to germinate and establish, and to increase complementarity between PFGs. No plots were weeded to allow colonization and natural assembly processes to occur after the sowing events.

Species belonging to three PFGs (legumes, forbs, grasses) were sown in each plot in 2012. Forbs included any non-leguminous, non-grass species. Grasses included members of the *Poaceae* family. Legumes are forbs of the *Fabaceae* family, which differ from species of other PFGs in their ability to fix atmospheric N_2.

Root sampling and data collection

Measuring roots is very laborious, such that we sampled a sub-set of plots from the field experiment. Since we expected the order of arrival of PFGs to affect root growth more than the sown diversity, we only sampled roots in the low diversity plots. Fine root length density was estimated in 2012 and 2014 in the G-first, L-first and control plots, using the ingrowth-core (IGC) method (Steen 1984; Hansson & Steen 1992; Steingrobe, Schmid & Classen 2000). We did not sample the F-first plots. Root samples were collected from 18 plots distributed on two different soil types. Each PFG order of arrival treatment was represented by six plots.

Using the IGC method, in each year, mesh bags (Polyamide fibre, length 45 cm, mesh size 1 mm, diameter 4 cm) were inserted into pre-drilled holes (diameter 5 cm) at an angle of 45° to the soil surface, covering a soil depth of approximately 30 cm. The drilled holes were protected by inserting PVC-tubes (diameter 4 cm) until the mesh bags were inserted. Four PVC tubes were installed in each plot with a distance of at least one meter between each bag, and one of the four IGC (mesh bags) were “activated” every two weeks (a PVC tube replaced by a mesh bag). All PVC tubes were covered with plastic caps. Each mesh bag was activated by filling it with soil, which was taken from an area adjacent to the experiment, with homogenous soil properties. The surface of this area was covered with an opaque plastic cover for several months to suppress any plant growth and to obtain soil material free from living roots. Before being used to fill the activated mesh bags, the soil material was collected from the topsoil (0-30 cm depth), air-dried, and 2 mm-sieved. Whenever a new IGC had to be activated, we repeatedly pulled out the PVC tube for a few centimetres, inserted a small quantity of soil material into the mesh bag, and compacted it. In order to achieve a soil density inside the mesh bags similar to that of the surrounding plot, the dry soil bulk density was determined previously and the appropriate soil quantity was filled into the mesh bags. A more detailed description of the ingrowth core method is provided by Steingrobe *et al.* (2000).

After activation, fine roots were allowed to grow inside the IGCs for a period of two weeks. Only one IGC at a time was active inside each plot, reflecting the fine root length density of a certain two week period (from here on called generation). Whenever one generation of IGCs was sampled, a reference soil core was taken from near the IGC in each plot for the examination of overall standing root length density. With this method, root decay inside the IGC during the 2 weeks exposure period is considered negligible, thus total root length inside the IGCs was considered to represent the total fine root length density for the given two-weeks period per soil volume of the cores (Steingrobe *et al.* 2001). After one generation was sampled, the next generation was activated to allow a continuous monitoring of fine root length density for eight weeks (from May to July of 2012 and 2014).

After two weeks of active exposure, mesh bags from each generation were pulled out and the samples were stored at -25°C until the roots were washed carefully over a 500 µm sieve. The total root length of each sample was determined by the line intersect method proposed by Newman (1966) and modified by Marsh (1971) and Tennant (1975). The root length (cm) was estimated using a 2 cm x 2 cm grid. Both standing (from the reference cores) and fine root length density (from IGCs) were calculated as the ratio between the measured total root length (cm) and the soil core volume (cm³). For large root samples, we measured the root length of a sub-sample, and then extrapolated the result for the entire sample collected from the field. In 2014 we also measured the standing and fine root biomass (as opposed to root length).

For each plot, the total fine root length density over a period of eight weeks was calculated as the sum of the values measured in each generation. From the reference cores we obtained the standing root length density, which was the mean value of the four generations. In order to verify which plant species from which PFGs were composing each plot, we used the cover estimation values measured in 2012 and the plant biomass values from 2014 (Weidlich *et al.* 2017).

Statistical analysis

In order to test any interaction between our main experimental factors with time, we first performed three-way ANOVAs, testing the effects of PFG order of arrival, soil type and year on fine and standing root length density. For standing root length density we found a significant interaction of PFG order of arrival and year (P=0.0035), while for fine root length density no significant interaction was found. A significant interaction between time and PFG order of arrival showed that the effects of the experimental treatments differed between the two years. Therefore, we analysed each year separately by performing two-ways ANOVAs testing the effects of PFG order of arrival and soil type (fixed factors) on standing and fine root length density, as well as the interaction between both fixed factors. The same was done for the standing and fine root biomass of 2014. In addition, we also compared generations (each year) by performing one-way ANOVAs testing the effect of (1) the generations within each order of arrival treatment and the effect of (2) order of arrival within each generation on standing and fine root length density. For all these analyses, when the null hypothesis was rejected (P<0.05), the mean treatment values were compared with a Newman and Keuls test performed with the R package agricolae (de Mendiburu 2015). All statistical analyses were performed using R 3.3.1 (R Core Team 2016).

RESULTS

Effects of PFG order of arrival and soil type on standing root length density

The size of the standing root length density (derived from the reference cores) in 2012 and 2014 was significantly affected by PFG order of arrival (P=0.0082; P=0.0268 - Fig. 3.1 and Supporting information 3.1), but not by soil type (Fig. 3.2). We did not find any significant interaction between the two factors in any of the two years.

More specifically, the results from 2012 showed that plots where grasses were sown first had higher standing root length density, compared to lower values in the plots where all PFG were sown at the same time (control) or when legumes were sown first (Fig. 3.1). At this time point, the treatments where grasses were sown first were dominated by grasses, while L-first and control treatments mainly consisted of plant species belonging to legumes (namely *Trifolium pratensis, Lotus corniculatus* and *Medicago sativa* - Fig. 3.1). A similar result was found in 2014, where the lowest standing root length density was also found in L-first treatments, but at this time point, it was significantly different from the other two treatments (Fig. 3.1). The standing root biomass measured in 2014 showed the same pattern (see Supporting information 3.2). The relative PFG dominance values showed that grasses dominated all treatments in 2014 (Fig. 3.1).

Looking at each generation separately (Supporting information 3.3), in 2012 the G-first showed the highest standing root length density in the third generation, and was the only treatment with significant differences across generations. In 2014 the three order of arrival treatments showed a significant decrease in standing root length density in the fourth generation, and L-first had the lowest values in the first generation.

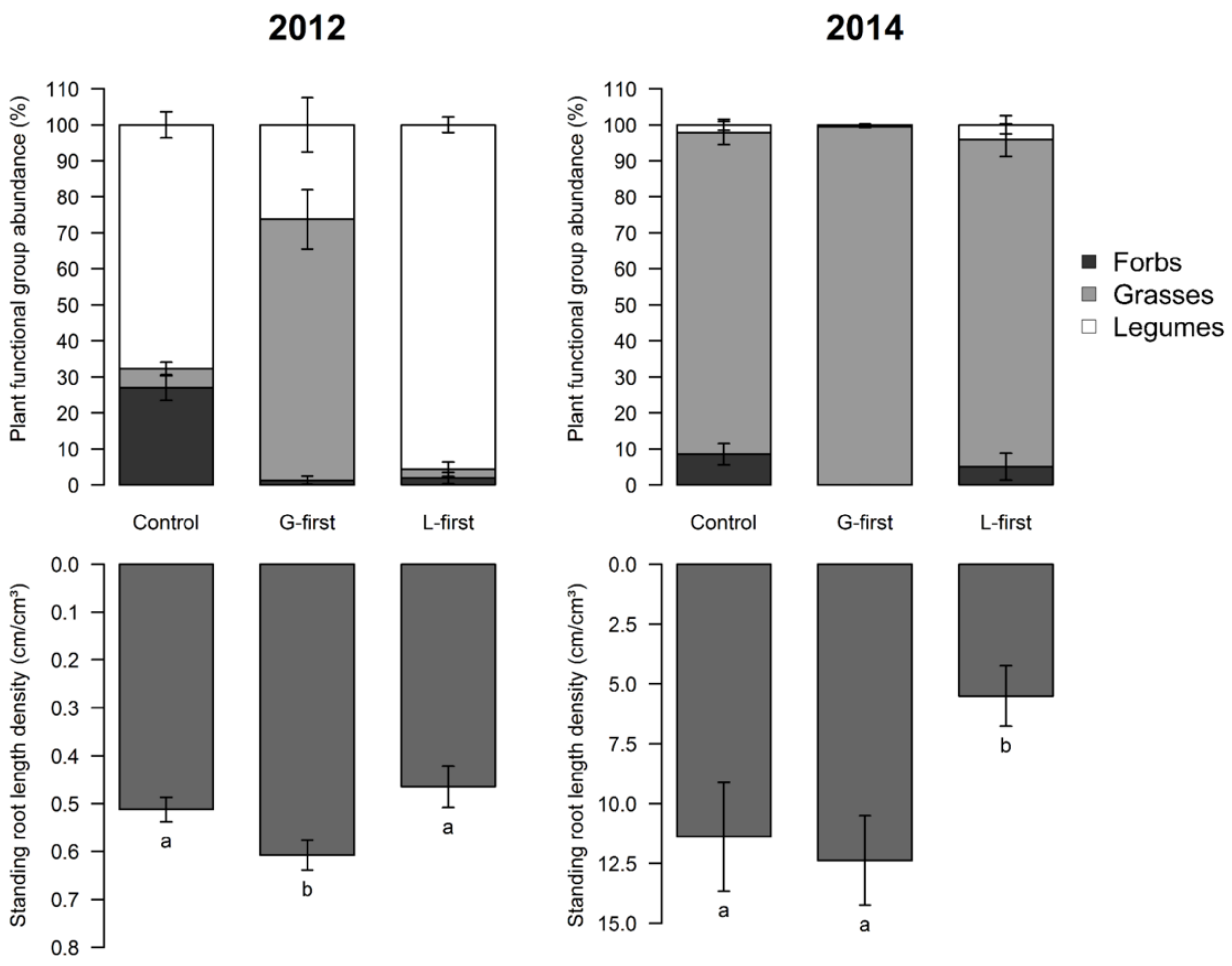


Figure 3.1. Influence of PFG order of arrival on PFG abundance and standing root length density in 2012 and 2014. In control plots, all PFG were sown at the same time. In the other plots, the PFG order of arrival was experimentally manipulated (G-first, grasses sown first; L-first, legumes sown first). Plant functional group abundance shows the percentage that each PFG represented in each treatment, obtained from aboveground cover estimations in 2012 and aboveground biomass in 2014. The values are means plus/minus one standard error of the mean. Different letters in root length density show significant differences between treatments (ANOVA followed by a Newman and Keuls test, $P < 0.05$).

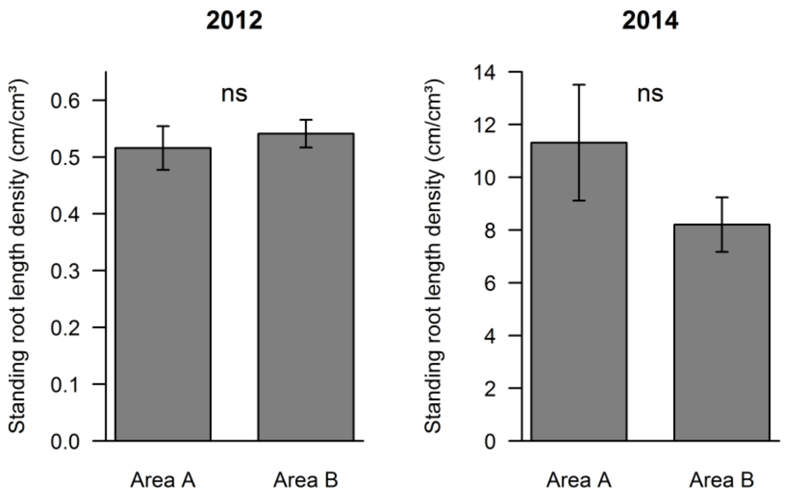


Figure 3.2. Influence of soil type on standing root length density in 2012 and 2014. Each area (A and B) was composed by a different soil type. The values are means plus/minus one standard error of the mean. Differences between treatments were not statistically significant (ns).

Effects of PFG order of arrival and soil type on fine root length density

The fine root length density (from the IGC) in both years was not affected by PFG order of arrival (Fig. 3.3a,b and Supporting information 3.1). In contrast, it was affected by soil type in 2012 (P=0.0296), with higher values in area A (Fig. 3.3c), but not affected by soil type in 2014 (Fig. 3.3d). The effect of the interaction between PFG order of arrival and soil type on fine root length density was never significant. The fine root biomass measured in 2014 showed higher values in the control compared to the G-first and L-first treatments (Supporting information 3.2).

Looking at each generation separately (Supporting information 3.3), no significant differences were found when we compared the order of arrival treatments within each generation in each year. Looking at each order of arrival treatment across generations, the fine root length increased over the four generations in 2012, while in 2014 fine root length varied across generations, with higher values in the fourth generation.

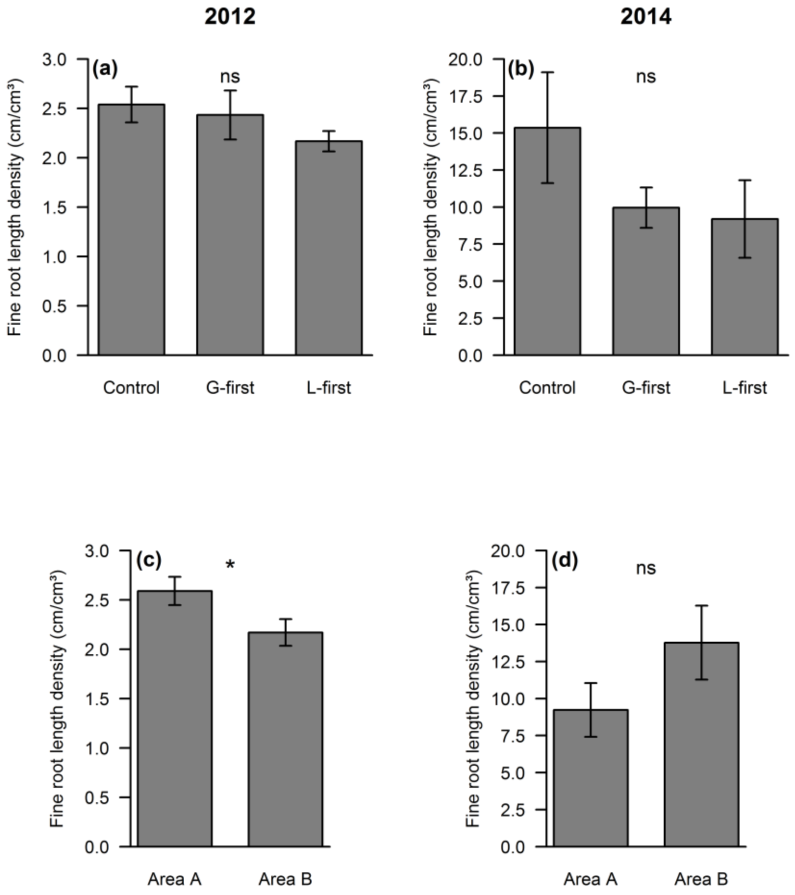


Figure 3.3. Influence of PFG order of arrival (a, b) and soil type (c, d) on fine root length density in 2012 and 2014. In control plots, all PFG were sown at the same time. In the other plots, the PFG order of arrival was experimentally manipulated (G-first, grasses sown first; L-first, legumes sown first). The values are means plus/minus one standard error of the mean. Significant differences ($P<0.05$) between treatments are represented by a star (*), and *ns* when not significant (ANOVA followed by a Newman and Keuls test, $P < 0.05$).

DISCUSSION

Our results helped to positively answer our first question, since priority effects created by manipulating PFG order of arrival had a significant effect on standing root length density. In the first year, treatments where grasses were sown first promoted higher values, while when legumes were sown first and all species were sown at the same time lower values were found. The species composition of PFGs found aboveground can explain this, since the L-first and control plots were dominated by legumes, whereas the G-first plots were dominated by grasses. This is possibly the reason why control and L-first plots had a lower standing root length density, since legumes normally invest less in roots and more in shoots, contrarily to grasses that have more extensive root systems (Körner *et al.* 2008; Poorter *et al.* 2015). This illustrates differences in belowground biomass allocation patterns and growth rates of legumes and grasses previously found by others in biodiversity experiments (where all species were sown at the same time) (Gastine, Scherer-Lorenzen & Leadley 2003; Ravenek *et al.* 2014). In these biodiversity experiments, legumes often get a head-start because they grow faster than grasses initially, but grasses become more dominant over time (both aboveground and belowground). Interestingly, in 2014, even though the PFG dominance of this year was different from 2012, with strong grass dominance in all the three treatments, we still found that the L-first treatments presented a lower standing root length density and standing root biomass. In addition, differences between L-first and other plots were even stronger in 2014. This indicates some form of priority effect occurring belowground in the L-first plots, where grass dominance in the vegetation at this time point (starting in 2013) did not translate into a catching up of root productivity in these plots. Intriguingly, despite the changes in species composition over time, the signal of sowing legumes first is even stronger belowground in the third year.

Competition aboveground is particularly asymmetrical (Weiner 1990), but whether or not belowground competition is asymmetrical in natural ecosystems is still unclear (Frank *et al.* 2010). In our experiment, the already established aboveground biomass from plants of the PFGs sown first was cut before the second sowing, while leaving belowground plant parts intact. Thus, we eliminated aboveground asymmetric competition aboveground. Nevertheless, species of the PFGs sown first continued to stay dominant in their respective treatments in the first (2012) and second (2013) year (Weidlich *et al.* 2017). A possible explanation for this could be that a once established root system has a competitive advantage (Brouwer 1983; de Kroon, Mommer & Nishiwaki 2003) particularly when most of the competitors (from other PFGs sown later) still have to establish a root system. The study of de Kroon *et al.* (2003) found that when aboveground plant parts had been cut, species which were sown first regenerated more quickly from disturbance than plants from PFGs sown later. In our experiment, however, in 2014, the community composition of all treatments was mainly dominated by grasses. Since this group of plant normally invest more in roots than in shoots, one could expect no differences in belowground development at this time point. But interestingly, even the PFG composition in the plots was so different between 2012 and 2014, the same pattern of root length was found in both time points.

The very few studies testing priority effects on root length or biomass (alongside other variables) also found an effect of plant order of arrival on root performance. Körner *et al.* (2008) found the same patterns as we found in our experiment. Sowing different functional groups at different times led to higher aboveground and lower belowground biomass when legumes were sown first, suggesting that species sown after the legumes may have a better chance of establishing as a result of the smaller root systems of legumes as well as via nitrogen (N) facilitation (which can include N sparing). In a greenhouse experiment, Sikes *et al.* (2016) introduced grasses and fungal species before others and found that small differences in early arrival history during the first two weeks of assembly were sufficient to affect plant performance for six months. Moore & Franklin (2012), found in a controlled experiment that root biomass of established species was reduced after water stress, but this outcome was dependent of priority effects and species identity. This provides evidence of the impacts of order of arrival on subsequent physiological and competitive ability of plants.

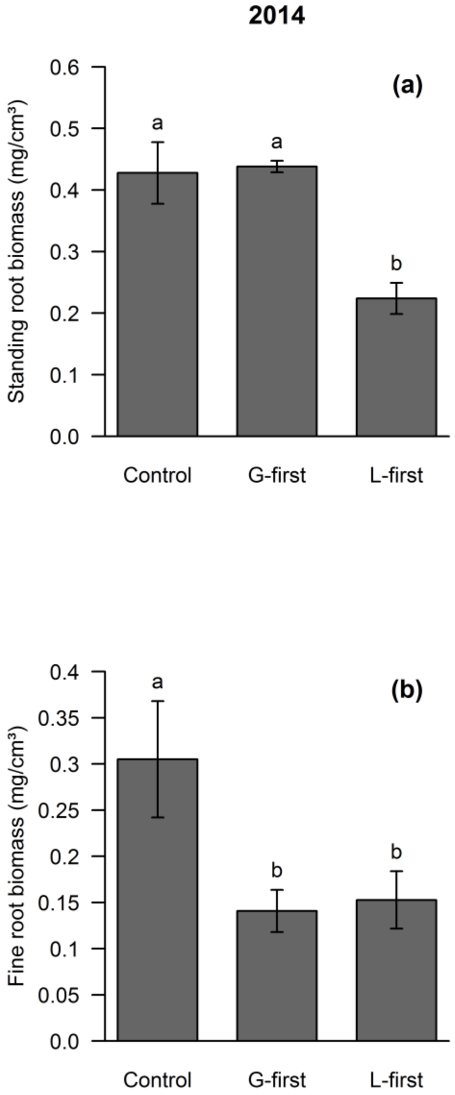
In regard to our second question, our results did not show that priority effects were modulated by soil type. We did not find an influence of soil type on standing root length density in any of the analysed years. On the other hand, there was an effect of soil type on fine root length density in 2012, where soil type of area A promoted higher root length than area B. In 2014, the opposite trend was found, with higher fine root length density in area B, but results were not significant. In a previous study in the same field experiment, we showed that area A with less fertile soil had a lower aboveground biomass but a higher number of species, while area B with more fertile soil had a higher biomass and lower number of species (Weidlich *et al.* 2017). Testing how timing of plant species arrival and soil nutrient interact to shape plant community, Kardol *et al.* (2013) found that arrival timing depends on resource availability, and that at high nutrient supply early arriving species grew quickly and reduced establishment of late arriving species. Soil N did not differ between the order of arrival treatments (Supporting information 2.4), which does not allow us to conclude that the lower root values found in the L-first plots may be related to differences in soil fertility. We also cannot argue that the lower root production in L-first plots in 2014 is due to changes in root allocation in grasses in response to soil fertility, because if this were the case, all treatments would have low root length density, since by 2013 all plots were dominated by grasses. In addition, even though grasses dominated all the treatments in 2014, we cannot know that the roots belonged mostly to grass species, since we could not differentiate roots of different species or functional groups (as we could do with the shoots).

Our study showed that grasses and legumes performed very differently as drivers of grassland assembly. It also showed that to what extent grasses and legumes drove assembly did depend on when they arrived in relation to other functional groups. Since European grasslands are extremely endangered due to both, intensive agriculture and land abandonment, any incentive for farmers to manage grasslands to improve productivity whilst maintaining diversity would be a gain for species conservation and in restoration. Our experiment provides evidence that sowing legumes first may be a good method for increasing aboveground biomass (if not belowground investment) whilst maintaining diversity of central European grasslands. Depending on the goal of a restoration project, sowing legumes first could foster lower root productivity (and provide more space and N sparing for other plants), versus higher investment in shoot biomass. However, if the overall goal of a project would be to foster soil carbon storage, then sowing legumes first would probably not be a benefit, depending on how generalizable our results prove to be, based on outcomes at other sites. Results from our field experiment showed sporadic priority effects aboveground (Weidlich *et al.* 2017), while in this study we found continuous priority effects belowground. On the other hand De Deyn *et al.* (2011) showed that the amount of C and N was increased in a long-term restoration practices especially when a legume was added to the system. This may be because legumes support a great microbial activity such that these plants would not depend as much on investing in roots to take up resources, and would allocate more C belowground to support the soil microbial community. Overall we found in our study strong evidence that sowing legumes first created a priority effect belowground that was found in the first and third year of this field experiment, even though the functional group dominance was different in each of the studied years. Thus, we can infer that belowground effects may be key drivers during the creation of priority effects, since these priority effects belowground were consistent over time.

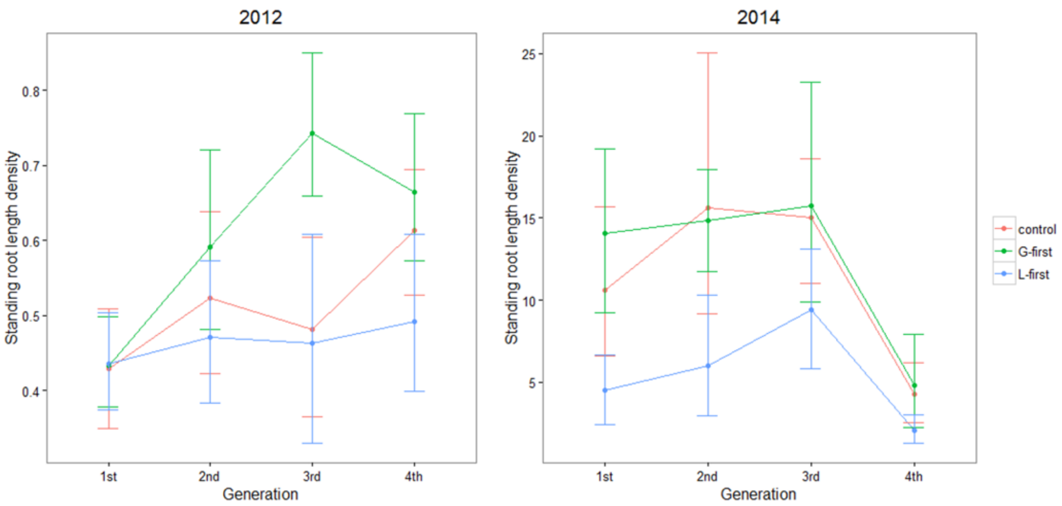
SUPPORTING INFORMATION CHAPTER 3

Supporting Information 3.1. Two-way ANOVA results showing the effects of plant functional groups order of arrival, soil type (areas A and B) and interaction on standing and fine root length density per year. Significances (P<0.005) are highlighted in bold.

		2012					**2014**				
Response	Factors	df	SS	MS	F	P	df	SS	MS	F	P
Standing root length density	Order or arrival	2	0.08455	0.04227	6.34425	**0.00822**	2	165.41400	82.70700	4.96383	**0.02686**
	Soil type	1	0.00386	0.00386	0.57899	0.45657	1	43.38300	43.38300	2.60372	0.13258
	Arrival*Soil	2	0.04602	0.02301	3.45341	0.05378	2	12.79600	6.39800	0.38399	0.68921
	Residuals	18	0.11994	0.00666			12	199.94300	16.66192		
Fine root length density	Order or arrival	2	0.58490	0.29245	1.53454	0.24245	2	135.31000	67.65500	1.70936	0.22220
	Soil type	1	1.06260	1.06260	5.57568	**0.02969**	1	93.05000	93.05000	2.35098	0.15110
	Arrival*Soil	2	0.66800	0.33400	1.75257	0.20163	2	0.36000	0.18000	0.00455	0.99550
	Residuals	18	3.43040	0.19058			12	474.95000	39.57917		

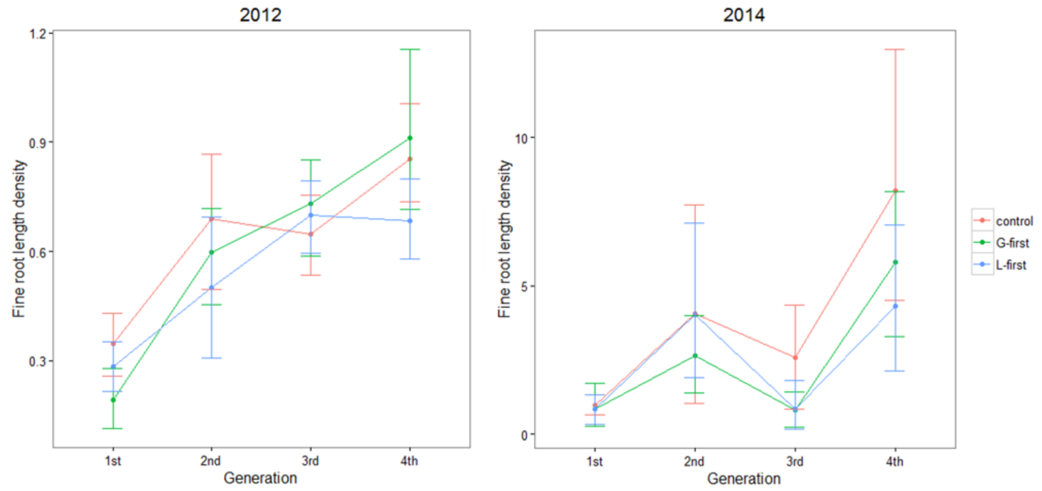


Supporting Information 3.2. Influence of PFG order of arrival on standing root biomass (a) and fine root biomass (b) in 2014. In control plots, all PFG were sown at the same time. In the other plots, the PFG order of arrival was experimentally manipulated (G-first, grasses sown first; L-first, legumes sown first). The values are means plus/minus one standard error of the mean. Different letters in root length density show significant differences between treatments (ANOVA followed by a Newman and Keuls test, $P < 0.05$).



Factor	P values			
Order of arrival	**1st gen**	**2nd gen**	**3rd gen**	**4th gen**
	0.9955	0.397	**0.0106**	0.0821
Generation	**L-first**	**G-frist**	**Control**	
	0.9213	**0.0023**	0.1305	

Factor	P values			
Order of arrival	**1st gen**	**2nd gen**	**3rd gen**	**4th gen**
	0.0369	0.0822	0.2503	0.2156
Generation	**L-first**	**G-frist**	**Control**	
	0.0333	**0.032**	**0.0432**	



Factor	P values			
Order of arrival	**1st gen**	**2nd gen**	**3rd gen**	**4th gen**
	0.0557	0.3893	0.6531	0.1894
Generation	**L-first**	**G-frist**	**Control**	
	0.0004	**<0.001**	**0.0005**	

Factor	P values			
Order of arrival	**1st gen**	**2nd gen**	**3rd gen**	**4th gen**
	0.9567	0.731	0.1567	0.3621
Generation	**L-first**	**G-frist**	**Control**	
	0.0413	**0.002**	**0.0338**	

Supporting Information 3.3. Influence of PFG order of arrival per generation on standing and fine root length density in 2012 and 2014. In control plots, all PFG were sown at the same time. In the other plots, the PFG order of arrival was experimentally manipulated (G-first, grasses sown first; L-first, legumes sown first). The values are means plus/minus one standard error of the mean. Tables below each graph show the P values of one-way ANOVAS.

CHAPTER 4

Paper accepted in January 2017

Methane yield of biomass from extensive grassland is affected by compositional changes induced by order of arrival

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D Popp, P von Gillhaussen, EWA Weidlich, H Sträuber, H Harms, VM Temperton (2017). GCB Bioenergy.

ABSTRACT

Low input grassland biomass from marginal and other slightly more fertile sites can be used for energy production without competing with food or fodder production. The effect of grassland diversity on methane yield has received some attention, but we do not know how community assembly may affect methane yield from grassland biomass. However, methane yields determine the potential economic value of a bioenergy substrate. Hence, a better understanding of how plant community assembly affects methane yield would be important. We measured biomass production and methane yield in the second year of a grassland field experiment which manipulated the order of arrival of different plant functional groups (forbs, grasses or legumes sown first and all sown simultaneously) and sown diversity (9 vs. 21 species). The order of arrival of the plant functional groups significantly determined the relative dominance of each group which in turn mainly explained the variance in aboveground biomass production. Differences in area-specific methane yields were driven by differences in biomass production and which plant functional groups dominated a plot. When grasses were sown first, legumes and grasses co-dominated a plot and the highest area-specific methane yield was obtained. Overall, the results indicate that altering the order of arrival affected the community functional and species composition (and hence methane yields) much more than sown diversity. Our study shows that a combined use of positive biodiversity effects and guided plant community assembly may be able to optimize methane yields under field conditions. This may allow a guided, sustainable and lucrative use of grassland biomass for biogas production in the future.

INTRODUCTION

Permanent species-rich grasslands can be valuable sources of biomass for biogas production. Indeed, biomass from grasslands has been increasingly used for energy provision in recent years (Prochnow *et al.* 2009) despite maize remaining dominant as a dedicated energy crop. If certain species compositions by virtue of their chemical composition are favorable for anaerobic digestion, methane yields can be optimized (Prochnow *et al.* 2009; Khalsa *et al.* 2014). Since biomass composition is more similar in species belonging to the same plant functional group (PFG), the presence and abundance of certain PFG affect the methane yield to a large extent (Herrmann *et al.* 2014; Stinner 2015).

Van Meerbeek *et al.* (2014) found a large range of annual biomass yield of low input high diversity systems including grasslands. In detail, many long-term biodiversity-ecosystem functioning (BEF) experiments, especially in grasslands, showed that higher richness of plant species and functional groups leads to higher plant productivity, due to positive biodiversity effects (Hector & Bagchi 2007; Marquard *et al.* 2009; Picasso *et al.* 2011). Van Meerbeek *et al.* (2015) studied the energy potential of different low input high diversity systems including grasslands. They found that grasslands had the highest energy efficiencies during anaerobic digestion and observed a correlation between biogas yield and functional group composition. However, it is still barely known how an increase in productivity as a consequence of higher species richness affects area-specific methane yields. Khalsa *et al.* (2014) studied effects of species richness and functional group composition within the Jena Experiment (Roscher *et al.* 2004) on biomethane production and area-specific methane potential. They found a positive effect of species richness and presence of legumes on area-specific methane potential. However, methane yields were only calculated from concentrations of chemical constituents of the substrates.

In most biodiversity experiments the diversity treatments were sown at the same time, and then plots were weeded to maintain the desired diversity gradient, therefore natural assembly did not occur. There is evidence however, that positive diversity effects also occur within a natural assembly context (Bullock *et al.* 2007). Bullock *et al*. (2007) tested the effect of sowing high and low diversity restoration seed mixtures on ex-arable land and found that sown diversity (after one sowing event) had effects on both productivity and diversity which persisted over many years.

The order of arrival of species can also be decisive for community assembly in that the plant species which arrive first at a site can significantly affect further assembly, with knock-on effects on diversity and biomass productivity (Diamond 1975; Martin & Wilsey 2012). Such so-called priority effects can drive species and functional diversity from the very early stages of a plant community (Chase 2003; Körner *et al.* 2008; Vaughn & Young 2010; Plückers *et al.* 2013).

In recent experiments under controlled conditions the order of arrival of PFG was directly manipulated. These studies found that the ensuing species richness and productivity of the communities was largely contingent upon such priority effects created by order of arrival

(Körner *et al.* 2008; von Gillhaussen *et al.* 2014). Additionally, it was found, that legumes sown before grasses and forbs resulted in more productive communities aboveground (von Gillhaussen *et al.* 2014) and less productivity belowground (Körner *et al.* 2008). These results open up the possibility of using priority effects to steer a community's development towards desired trajectories of ecosystem function and possibly diversity. Based on the fact that the plant functional identity plays a key role for methane yields within a biogas setting (Herrmann *et al.* 2014; Stinner 2015), knowing more about how to direct plant functional composition towards optimal methane yield would be a valuable gain for the sustainable economic use of restored species-rich grasslands.

Knowledge from ecological experiments may allow for more sustainable management of species-rich grasslands that are highly endangered in Europe either through agricultural intensification or land abandonment. If we find that a combined use of positive biodiversity and order of arrival effects (Temperton *et al.* 2016) shows positive results under field conditions, this may allow a sustainable and lucrative use of grassland biomass for biogas production. This would provide a win-win situation if aboveground productivity can be increased by both biodiversity and order of arrival effects whilst not diminishing biomass quality. This would also enhance the provision of a number of different ecosystem services especially in agro-ecosystems (Altieri 1999; Bullock *et al.* 2001b; Rey Benayas & Bullock 2012). However, in most of these ecological experiments the effects of either richness or order of arrival of species on aboveground-productivity and other ecosystems functions were investigated individually. The combined effects of sown species richness and order of arrival (priority effects) on ecosystem functions especially in a natural assembly were barely addressed whether under controlled or field conditions, but see Weidlich *et al.* (2016). Even less is known about how grassland methane yield may respond to sown diversity and the creation of priority effects.

We studied a mesotrophic grassland field experiment composed by central European native species adapted to relatively nutrient-rich soils. We analyzed methane yield in 2013 in a field experiment (the Priority Effect experiment; see Weidlich *et al.* (2017) for details), which was set up in 2012 to investigate the effects of two main factors: the order of arrival of PFG (either forbs, grasses or legumes sown first followed by the other two groups) and sown diversity (9 vs. 21 species) on grassland community assembly and ecosystem functions.

Biomass from the September harvest in 2013 (prior to the second mowing of the growing season) was anaerobically digested and its biomethane potential as well as area-specific methane yield was compared. The aim of this study was to test the following hypotheses:

1. Methane potential of biomass will differ depending on variations in species and functional group abundances induced by order of arrival of different PFG and sown diversity.
2. The dominance of legumes will positively affect both the aboveground biomass and the methane yield and therefore best results for area-specific methane yields will greatly depend on the species and functional composition.

MATERIALS AND METHODS

Experimental Setup and Design

We measured methane yields in the Priority Effect experiment, a grassland experiment in which aboveground biomass and community composition were monitored from 2012 to 2015, see (Weidlich *et al.* 2017). With this experiment the effects of sown diversity and order of arrival of PFG on aboveground biomass and methane yield were tested on two different soil types. The factor order of arrival of PFG had four levels, with forbs sown first (F-first), grasses sown first (G-first), legumes sown first (L-first) and all PFG sown at the same time (control). The factor sown diversity had two levels: low diversity (LD: 9 species) and high (HD: 21 species). Soil type A was classified as Stagnic Cambisol and soil type B as an Anthrosol according to the official German soil mapping guidelines (Sponagel 2005).

Species selection and treatments

The seed mixtures consisted of typical central European grassland species so that the target plant community was a semi-natural, species-rich, mesotrophic grassland. Species were classified into three PFG (non-legume forbs, grasses and legumes) which differ significantly in functional and morphological traits (based on Roscher *et al.* 2004). In total, a fixed set of 21 common grassland species (7 forbs, 7 grasses and 7 legumes) was selected for high diversity communities. A fixed subset of 9 species (3 forbs, 3 grasses and 3 legumes) was selected to represent low diversity communities (see Weidlich *et al.* 2017).

The PFG order of arrival was created by sowing the species of one PFG first on 19.04.2012 (or all at the same time as control) and the other species of the remaining PFG at the same time on 31.05.2012. Before the second sowing, all plots were mown at a cutting height of 30 mm, to reduce initial aboveground competition and to allow subsequently sown species to establish well. None of the plots were weeded after sowing to allow assembly close to natural dynamics. All plots were mown twice per growing season (in early June and early September after biomass-sampling) as typical for such grasslands and plant material was removed from the plots.

Sampling and Data Collection

The core area (3.5 × 3.5 m) within every plot (4 × 4 m) was used for species specific cover assessments and biomass harvesting. Plant cover per species (community composition) and species richness (total number of species present) were estimated prior to each harvest. Cover assessments were performed using a modified decimal cover estimation method following Braun Blanquet (Londo 1976). Total community cover was higher than 100% as the canopy was multilayered and three-dimensional. Total aboveground biomass was measured using two randomly distributed 0.1 m^2 rectangles (20 × 50 cm) within the core areas of each plot, avoiding areas where previous sampling was done. All aboveground plant material within the rectangle was cut approx. 5 cm above soil surface and samples were dried at 70°C for two days before weighing.

Biomethane potential

We measured biomethane potentials of grassland aboveground biomass harvested in September 2013 from eight low diversity plots from both soil types with forbs, grasses or legumes sown first and all PFG sown simultaneously (first replicate of each treatment). In addition, to get a slight handle on how the high and low diversity treatments may have affected the methane potentials, we sampled aboveground biomass from two high diversity plots (one of each soil type, first replicate of each treatment) on which all PFG were sown simultaneously (control plot). Batch experiments were performed using triplicate 400-g assays in an Automated Methane Potential Test System (AMPTS II, Bioprocess Control, Sweden) in accordance with VDI 4630 (VDI 4630, 2006). Mixtures of digestates from a farm-scale and a lab-scale biogas reactor served as inocula with total solids (TS) contents of 3.8 - 4.4% and volatile solids (VS) contents of 65 - 69%$_{TS}$. The TS and VS contents were determined as described by (Sträuber, Schröder & Kleinsteuber 2012). Inocula were stored at 37°C for at least five days to allow for degassing prior to the biomethane potential tests.

Dried plant biomass was digested under anaerobic conditions with an inoculum to substrate ratio of 2:1 (VS basis) according to VDI 4630 (VDI 4630, 2006). Accordingly, 6.2 - 8.1 g of substrate and 391 - 394 g of inoculum were added to 500-mL bottles. Duplicate negative controls were implemented by digesting only the inoculum, and these reactors were filled up with distilled water to 400 g in total. Furthermore, microcrystalline cellulose (2.5 g) was digested as reference substrate together with the inoculum in a single bottle. Inocula showed sufficient activity as at least 90% of the theoretical methane yield of cellulose (VDI 4630, 2006) was achieved. The AMPTS system was set up and operated as described previously (Popp *et al.* 2015). After 30 days, the daily methane production was lower than 1% of the total methane production and experiments were stopped on day 35. The area-specific methane yield for the second cut (September 2013) was calculated as product of aboveground biomass (g_{TS} m^{-2}) and biomethane potential (L_N CH_4 g_{VS}^{-1}) taking into account the VS content.

Statistical Analyses

Response variables were biomethane potential as well as area-specific methane yield for the plots sampled for methane yield and the relative dominances (RD) of the PFG and community aboveground biomass of all plots. Beside order of arrival and sown diversity, soil type was included in the analyses as factor to test for interactions with the other two factors. Average values for both soil types were given when differences of a response variable for both soil types and interactions with other factors were not statistically significant. Statistical analyses were performed with R version 3.2.0 using the packages ‘lsr’, ‘stats’ and ‘car’. Normal distributions and homogeneity of variance of variables were tested by Shapiro-Wilk and Levene’s test, respectively. There was no collinearity between RD of the

PFT according to the variance inflation factors which were calculated using the R package ‘usdm’.

RD of PFG were analyzed by three-way multivariate ANOVA (analysis of variance) for effects of order of arrival of PFG, sown diversity and soil type. As soil type had no significant influence on RD of PFG, results are given as average values for both soil types (Fig. 4.1, Table 4.2). Furthermore, effects of the factors order of arrival and sown diversity on the RD of the individual PFG were tested by two-way univariate ANOVA. Observed species richness was analyzed by ANOVA for effects of order of arrival and sown diversity. Community aboveground biomass was analyzed by three-way ANOVA for the effects of the RD of the PFG (Table 4.1). Biomethane potentials and area-specific methane yields were analyzed by three-way ANOVA for effects of the order of arrival of PFG and sown diversity. In addition to these two effects, effects of soil type on area-specific methane yields were analyzed. Furthermore, effects of RD of PFG on biomethane potentials and area-specific methane yields were studied by three-way ANOVA (Table 4.1). Effect sizes were given as (partial-) η^2 representing the power of the analysis based on the replication. High values denote high percentages of variance of the response variable explained by the source variable. Normality and homogeneity of residuals of the models were tested by qq-plots and by plotting the residuals against the fitted values.

RESULTS

In this study, when we sampled in September 2013 low diversity plots for methane potential determination, legumes dominated all treatments except when forbs were sown first (Fig. 4.1 and Table 4.2). On high diversity plots the PFG which was sown first dominated and when all PFG were sown simultaneously grasses were most abundant (Fig. 4.1).

In detail, the order of arrival and sown diversity affected the RD of the three different PFG in the community composition in September 2013 (MANOVA with Pillai’s trace: order of arrival V = 1.12, $F_{9,168} = 11.2$, $p < 0.001$ and sown diversity V = 0.36, $F_{3,54} = 10.1$, $p < 0.001$), see Fig. 4.1. However, only the factor order of arrival significantly affected the RD of each PFG as revealed by separate univariate ANOVA (RD forbs: $F_{4,59} = 16.8$, $p < 0.001$; RD grasses: $F_{4,59} = 29.9$, $p < 0.001$; RD legumes: $F_{4,59} = 7.0$, $p < 0.001$).

The sown diversity had a small but significant influence on the RD of forbs ($p = 0.015$, $\eta^2 = 5\%$). Grasses dominated on high diversity plots when grasses were sown first or all PFG were sown simultaneously (order of arrival: $p < 0.001$, $\eta^2 = 53\%$). On low diversity plots RD of grasses was lower than on high diversity plots (sown diversity: $p < 0.001$, $\eta^2 = 14\%$, Fig. 4.1). RD of legumes was high when legumes were sown first as well as on all low diversity plots except when forbs were sown first (order of arrival: $p < 0.001$, $\eta^2 = 32\%$) with sown diversity having no effect ($p = 0.556$, Fig. 4.1). Furthermore, as expected species richness was strongly determined by sown diversity ($p < 0.001$, $\eta^2 = 52\%$). On average 13 (±0.4 standard error) and 8 (±0.4) species were found on HD and LD plots, respectively (compared to 21 and 9 sown species). In contrast, order of arrival had only a small influence on species richness ($p = 0.011$, $\eta^2 = 8\%$).

The community aboveground biomass had a mean of 574 g_{TS} m^{-2} in September 2013 with a large range from 229 g_{TS} m^{-2} to 951 g_{TS} m^{-2} (Fig. 4.2a). Variances in the aboveground biomass can partly be explained by the RD of the PFG (Table 4.1). However, only the RD of legumes explained the observed aboveground biomass significantly.

Biomethane potentials

In this study the effects of order of arrival (eight low sown diversity plots) and of sown diversity (two control plots with all PFG sown at the same time) on biomethane potentials were explored. Biomethane potentials, given as feedstock-specific methane yield, had a range from 231 to 278 mL_N g_{VS}^{-1} across all sub-sampled plots (Fig. 4.2b and Table 4.2). No significant differences between biomethane potentials due to order of arrival of PFG (as measured in the low sown diversity plots, $p = 0.236$) or due to sown diversity (control plots, $p = 0.810$) were found. However, the RD of PFG from each plot determined the biomethane potentials significantly (Table 4.1). In detail, the RD of forbs, legumes and the interaction of the three PFG had significant effects on the biomethane potential ($p < 0.05$).

Table 4.1. ANOVA table of aboveground biomass, biomethane potentials and area-specific methane yield as explained by the relative dominance (RD) of forbs, grasses and legumes (as well as their interactions) growing in the sampled plots in September 2013. Significant factors ($p < 0.05$) are highlighted in bold. Effect size partial-η^2 shows the power of the analysis based on the replication. High values denote high percentages of variance of the response variable explained by the source variable.

Response	Source: Relative dominance	df	SS	MS	F	*p*	η^2
Above-ground biomass	**Legumes**	1	139633	139633	4.484	**0.039**	6.5%
	Grasses	1	25027	25027	0.804	0.374	0.8%
	Forbs	1	7212	7212	0.232	0.632	0.4%
	Legumes × Grasses	1	19569	19569	0.628	0.431	1.3%
	Legumes × Forbs	1	655	655	0.021	0.885	0.4%
	Grasses × Forbs	1	14898	14898	0.478	0.492	0.7%
	Legumes × Grasses × Forbs	1	54875	54875	1.762	0.190	2.7%
	Residuals	56	1743960	31142			86.9%
Biomethane potential	**Forbs**	1	840	840	24.100	**0.008**	36.5%
	Legumes	1	749	749	21.500	**0.010**	32.6%
	Grasses	1	168	168	4.818	0.093	7.3%
	Legumes × Forbs	1	10	10	0.299	0.613	0.5%
	Grasses × Forbs	1	82	82	2.351	0.200	3.6%
	Legumes × Grasses	1	42	42	1.201	0.335	1.8%
	Legumes × Grasses × Forbs	1	355	355	10.194	**0.033**	15.4%
	Residuals	4	139	35			6.1%
Area-specific methane yield	**Forbs**	1	7644	7644	138.133	**0.000**	41.5%
	Grasses	1	4516	4516	81.602	**0.001**	24.5%
	Legumes	1	4110	4110	74.276	**0.001**	22.3%
	Grasses × Forbs	1	1479	1479	26.726	**0.007**	8.0%
	Legumes × Forbs	1	172	172	3.101	0.153	0.9%
	Legumes × Grasses	1	4532	4532	81.905	**0.001**	24.6%
	Legumes × Grasses × Forbs	1	2285	2285	41.291	**0.003**	12.4%
	Residuals	4	221	55			1.2%

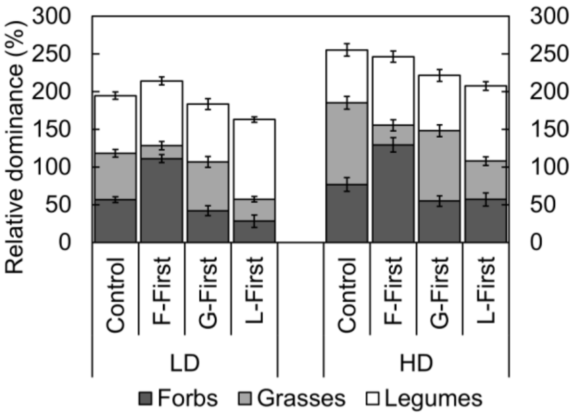


Figure 4.1. Mean relative dominances (n = 8, ±SE) of plant functional groups (PFG) forbs, grasses and legumes found growing in September 2013 depending on their order of arrival (control: all PFG sown simultaneously, F-first: forbs sown first, G-first: grasses sown first, L-first: legumes sown first) and on sown diversity (LD: low diversity, HD: high diversity). Note that the sum of RD is higher than 100% as plant species overlapped within canopies. One can see that in both HD and LD treatments the PFG that was sown first in 2012 still dominated the vegetation in September 2013 (except for LD G-first where legumes co-dominated with grasses).

Table 4.2. Mean relative dominances (RD) of plant functional groups (PFG) in plots in September 2013, mean biomethane potentials and mean area-specific methane yields in relation to sown diversity and order of arrival of PFG. Note that the sum of RD is higher than 100% as plant species overlapped within canopies. Means with same letter (superscript) are not significantly different.

Sown diversity	Order of arrival	RD forbs (%, ±SE)	RD grasses (%, ±SE)	RD legumes (%, ±SE)	Biomethane potential (L_N CH_4 g_{VS}^{-1}, ±range)	Area-specific methane yield (L_N CH_4 m^{-2}, ±range)
Low diversity	F-first	111 (±10)^{a}	16 (±5)^{a}	86 (±5)^{a,b}	234 (±2)^{a}	106 (±13)^{a}
	G-first	42 (±15)^{b,c}	65 (±7)^{b}	77 (±7)^{b}	250 (±5)^{a}	148 (±17)^{a}
	L-first	28 (±11)^{c}	29 (±8)^{a}	106 (±4)^{a}	256 (±13)^{a}	101 (±3)^{a}
	Control	57 (±5)^{b,c}	62 (±4)^{b}	77 (±5)^{b}	262 (±16)^{a}	121 (±8)^{a}
High diversity	Control	77 (±12)^{a,b}	109 (±9)^{c}	70 (±8)^{b}	264 (±1)^{a}	114 (±31)^{a}

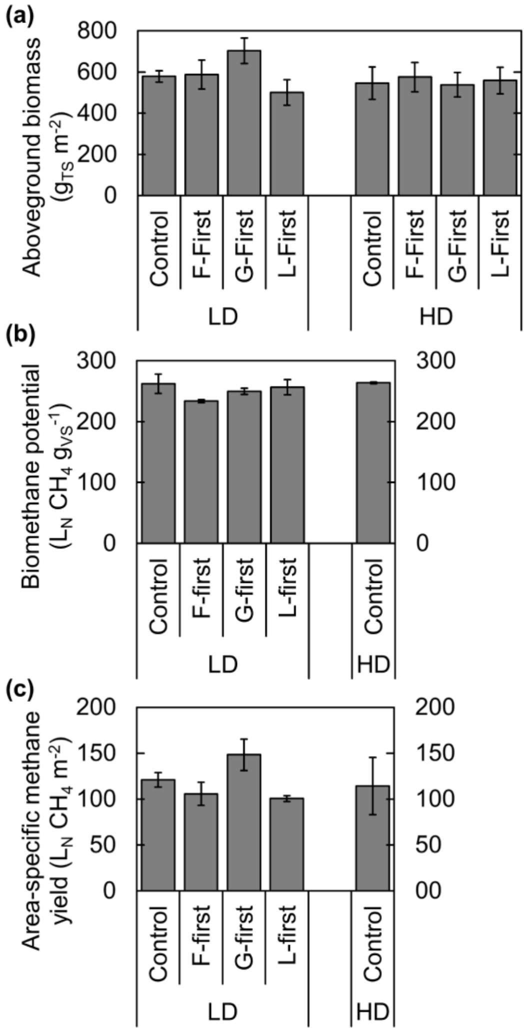


Figure 4.2. (a) Mean aboveground biomass of all plots harvested in September 2013 (n = 8, ±SE), (b) mean biomethane potentials (n = 2, ±range), as well as (c) mean area-specific methane yield of sub-sampled plots (n = 2, ±range) according to the order of arrival of PFG (control: all PFG sown simultaneously, F-first: forbs sown first, G-first: grasses sown first, L-first: legumes sown first) and sown diversity (LD: low diversity, HD: high diversity).

Area-specific methane yield

Aboveground biomass and biomethane potential were taken into account to evaluate methane yield per area unit. The area-specific methane yields had a mean of 119 L_N CH_4 m^{-2} and a large range which was solely due to the variations in aboveground biomass ($p < 0.001$, $\eta^2 = 97\%$) and not due to biomethane potentials ($p = 0.600$, $\eta^2 = 1.8\%$). The variance of the area-specific methane yields can be explained by order of arrival ($p = 0.168$, $\eta^2 = 23\%$), sown diversity ($p = 0.135$, $\eta^2 = 14\%$) and soil type ($p < 0.05$, $\eta^2 = 27\%$) based on the effect size rather than significance. Biomass from soil B had a slightly higher methane yield than from soil A (127 (±10) L_N CH_4 m^{-2} compared to 110 (±9) L_N CH_4 m^{-2}). This reflects the higher aboveground plant biomass results, which however were not found to be significantly different. As the factor soil type was not the focus of this study, mean values for both soil types were given hereafter and in figure 4.2c as well as table 4.2. The highest area-specific methane yield of 148 L_N CH_4 m^{-2} was obtained from plots where grasses were sown first (Table 4.2 and Fig. 4.2c). When PFG were sown simultaneously (control) the mean area-specific methane yields were 121 L_N CH_4 m^{-2} and 114 L_N CH_4 m^{-2} at low and high sown diversity, respectively. Lowest area-specific methane yields were obtained from plots where forbs and legumes were sown first (106 and 101 L_N CH_4 m^{-2}, respectively).

Differences in the area-specific methane yield can also be explained by the RD of the PFG (Table 4.1). Most of the variance was explained by the RD of forbs, grasses and legumes as well as the interaction of grasses and forbs. Methane yields were higher when RD of legumes were between 70% and 77% and RD of grasses were as high as 62% – 109% (Table 4.2). Higher RD of legumes (>86%) and low RD of grasses (≤29%) resulted in lowest methane yields. Species richness had no significant effect on the area-specific methane yield.

DISCUSSION

The biomass production and methane yield data presented this study are from September 2013, when legumes were the most dominant functional group (see Fig. 4.1). In more detail, forbs were dominating in the F-first plots (both HD and LD levels) and grasses were dominating in control and G-first plots in high diversity plots. Our biomethane potential study shows clearly that the order of arrival affected the RD of specific PFG and this in turn significantly affected the aboveground biomass, the biomethane potential and the area-specific methane yield. Furthermore, we obtained an indication for an effect of sown diversity on the RD of PFG and hence, methane yields.

Our reported aboveground biomass production was well within the range described in the literature for similar grasslands consisting of typical European species (Bullock *et al.* 2007; Marquard *et al.* 2013). Henschell *et al.* (2015) reported an even greater range from 80 to 1070 g m^{-2} annual aboveground biomass production for low-input grasslands. However, the second cut usually yields less biomass than the first cut within a year (Amon *et al.* 2007; Khalsa *et al.* 2014). Biomethane potentials expressed as feedstock-specific methane yields represent the potential energy of a certain biomass which can be exploited by anaerobic digestion. Biomethane potentials found in this study were similar as reported for low-input conservation areas (Herrmann *et al.* 2014; Van Meerbeek *et al.* 2015) and as the theoretical potentials of low-input grassland (Corton *et al.* 2013) or lower than reported earlier for typical European grasslands and different meadows (Melts *et al.* 2013; Khalsa *et al.* 2014). Even though species compositions of the sub-sampled plots were different, no significant differences between biomethane potentials were found which is in contrast to other studies reporting clear differences (Herrmann *et al.* 2014; Khalsa *et al.* 2014). Significant differences between biomethane potentials were also found between single grass species (Seppälä *et al.* 2009; Mc Eniry & O'kiely 2013). Furthermore, Melts *et al.* (2014) observed a higher biomethane potential of grasses than of legumes and forbs. However, in our study herbs and legumes contributed to the biomethane potential as well and an averaging effect might occur when grassland communities of different composition are utilized for biogas production.

The aim of supplying biomass for biogas production is a maximized area-specific methane yield comprising biomass production and biomethane potential which are both influenced by many factors. Literature on area-specific methane yields of grassland communities is rare. Khalsa *et al.* (2014) reported yields of 56 to 111 L_N CH_4 m^{-2} which is low compared to our results. Mc Eniry & O'kiely (2013) and Seppälä *et al.* (2009) reported methane yields from 116 to 350 L_N CH_4 m^{-2} for single grass species which are similar or higher than yields obtained in this study. Area-specific methane yields were influenced strongly by biomass production of different plant functional groups and not by biomethane potentials, which is consistent with results from Khalsa *et al.* (2014).

Furthermore, Khalsa *et al.* (2014) described a positive correlation between legume abundance and area-specific methane yields. In turn, they found a negative correlation with the abundance of grasses. These correlations were linked to lower crude fiber and higher crude protein content of legumes compared to grasses. Lignocellulosic fibers are hard to degrade under anaerobic conditions in contrast to proteins thus lowering the biomethane potential (Klimiuk *et al.* 2010; Herrmann, Idler & Heiermann 2016). Furthermore, community biomass production is increased by legumes due to nitrogen fixation (Roscher *et al.* 2011b). This is supported here, in that we found that the RD of legumes and grasses positively affected area-specific methane yields by increased biomass production. Furthermore, this may be due to general differences in chemical composition of PFG (not measured in this study) or to competition for light between PFG leading to different fiber contents as hypothesized by Khalsa *et al.* (2014).

The sown diversity affected the ensuing species richness of the plots as well as the relative dominance of PFG. In contrast to other studies(Bezemer & Van Der Putten 2007; Bullock *et al.* 2007; Mangan *et al.* 2011; Khalsa *et al.* 2014) sown diversity did not affect aboveground biomass or area-specific methane yields.

The main driver of the methane potential and yield however, was clearly the indirect effect of assembly treatments on the relative dominance of the plant functional groups. Since the differences in methane yields are not large between the treatments where grasses were sown first and the other treatments (and in particular are not significantly different from the control) looking at the composition of the community is probably more important to methane yield than which PFG was sown first. The relative dominance of the PFGs that manage to establish in the grassland plots is actually what was driving the area-specific methane yield. According to our data, sown diversity seemed to have a minor influence on methane yield although it should be noted here that we only compared HD with LD in the assembly control plots (sown at the same time) such that a general conclusion here would require further research.

In our study at this time point (September 2013) highest area-specific methane yields were obtained when grasses were sown first and where grasses and legumes co-dominated. One should take into account however, that this result is only one time point in the experiment. If further studies also find the same pattern then one would have to consider the possible implications of this outcome in relation to restoration goals. A restoration process aiming at a high biodiversity might be different than aiming at a high methane yield. When grasses were sown first and highest methane yields were observed, grasses dominated and a lower species diversity compared to the control was observed which is not desired for a high biodiversity. Further research is necessary to see if biodiversity and bioenergy goals can be balanced by a specific restoration strategy.

Interestingly, we found that the assembly treatment determined the relative dominance of the plant functional groups and that the highest area-related methane yield was obtained when legumes and grasses co-dominated. These effects may persist for several years as observed by Bullock *et al.* (2007) and Plückers *et al.* (2013). However, in the Priority Effect experiment, on which this biomethane study is based, priority effects were not persistent over time (Weidlich *et al.* 2017). These conflicting results show that the assembly process needs to be further investigated. Priority effects might depend on the year of sowing, on impact of harvesting and weather conditions. According to our results, it is likely that grassland communities can be steered towards high area-specific methane yields by using priority effects. This seems to be a potentially valuable tool to maximize methane yields from grasslands. As feedstock-specific methane yields are secondary (own data and literature), efforts to optimize area-specific methane yields can be simplified by focusing on assembly and plant biomass production.

CHAPTER 5

Paper under Review in Plant and Soil

Neighborhood stories: role of neighbor identity, order of arrival and spatial location in legume and non-legume interactions

EWA Weidlich, VM Temperton, M Faget (submitted Plant and Soil)

ABSTRACT

Backgrounds and Aims: Interactions between plants can be both positive and negative (facilitation and competition). Although facilitative effects of having legume neighbors (focus on yield productivity) are well studied, a better mechanistic understanding on how legumes interact with non-legumes in terms of root distribution is needed. We tested the effects of neighbor identity, its spatial location, as well as the effects of plant order of arrival on above and belowground performance and root distribution.

Methods: We performed a rhizotron experiment in which we grew maize alone, with only a legume, only another grass, or with both species and tracked roots of the plant species using green and red fluorescent markers.

Results: Maize grew differently when it had a neighbour, with reduced performance when growing with wheat compared to alone. Growing with a legume generally equated to the same outcome as not having a neighbour. Roots grew towards the legume species and away from the wheat. Order of arrival affected aboveground traits to a certain extent, but its effects on maize roots were dependent on spatial location.

Conclusions: The identity of the neighbours, together with their spatial location, plays a key role in plant-plant interactions and their effects on performance, and order of arrival can modulate the outcome of these interactions.

INTRODUCTION

Interactions between plants play an important role in driving ecosystem functioning and providing ecosystem services (Roscher *et al.* 2005). Interactions can be either positive (facilitation), negative (competition) or simultaneously positive and negative, thus affecting community assembly (Callaway *et al.* 2002; Temperton *et al.* 2007; Brooker *et al.* 2008). Belowground competition can be stronger than aboveground, and causes a decrease of growth, fecundity or survival of plants (Casper & Jackson 1997). Recent research has shown that when plant individuals compete with one another, the identity of the neighbour is important (Semchenko *et al.* 2014). Indeed the presence of potential competitors for soil resources has been shown to stimulate changes in biomass allocation towards roots (Falik *et al.* 2003; Padilla *et al.* 2013).

Facilitation, whereby one plant species indirectly or directly creates conditions that benefit a neighbouring or subsequent plant (Connell and Slatyer 1977; Brooker et al. 2008), plays an important role in plant communities and affects ecosystem functioning (Roscher *et al.* 2011a). One form of facilitation, nurse plant facilitation, works via the creation of favourable microsite conditions for emerging seedlings so they can survive adverse conditions (Valiente-Banuet *et al.* 2006). Nitrogen (N) facilitation, in contrast, involves atmospheric N_2-fixing plant species (often legumes from the Fabaceae family), and works via an increase in the availability of N for neighbours of legumes (Schenk 2006; Temperton *et al.* 2007). Mechanisms of N facilitation include direct N transfer from the legume to neighbours (either via mycorrhizae or root exudation), N sparing and over longer time scales increased N availability via decomposition of legume tissues. N sparing occurs when non-legume neighbours profit from the spare N available in the soil that legumes are not taking up when they mainly rely on N fixed from the atmosphere (Temperton *et al.* 2007; von Felten *et al.* 2009).

Facilitation and competition can be sensitive to the order that species arrive in the system (Fukami 2015). Priority effects occur when species that arrive first in the system significantly affect the ones that establish later, thus affecting the trajectory of the community (Eriksson & Eriksson 1998; Fukami 2015; Vaughn & Young 2015). Priority effects are about “who” arrives when during assembly and succession and can be either inhibitory or facilitative (Connell and Slatyer 1977, Fukami 2015). A number of grassland experiments (both under controlled and field conditions) have found that sowing legumes before other functional groups created priority effects that influence ecosystem functioning (Körner et al. 2008; von Gillhaussen et al. 2014; Temperton et al. 2017, Weidlich et al. 2017). Fukami (2015) proposed two mechanisms of priority effects: niche pre-emption and niche modification. In the first mechanism first arrivals reduce the amount of resources available to other species, while in the second the early arriving species change the niches available in the site and in consequence the identity of the species that arrive later.

Experiments testing interactions between intercropping species has been the focus of many studies in agricultural science (Hauggaard-Nielsen & Jensen 2005; Hauggaard-Nielsen *et al.* 2008; Duchene, Vian & Celette 2017). However, they normally aim in the effects of intercropping on nutrient acquisition and yield production (Li *et al.* 1999, 2001, 2003; Fan *et* *al.* 2006; Neugschwandtner & Kaul 2014; Zhang *et al.* 2014). So far experiments testing root interactions involving a legume species have investigated the effects of fertilization and root interactions on agronomic traits (Zhang *et al.* 2013), methods to evaluate root competition in initial growth (Tosti & Thorup-Kristensen 2010), how root competition is affected by nitrogen supply at different levels (Andersen, Dresbøll & Thorup-Kristensen 2014; Ramirez-Garcia *et al.* 2014). Although facilitative effects of having legume neighbours are well studied, a better mechanistic understanding on how legumes interact with non-legumes in terms of root distribution is needed. The effects of the identity and spatial location of neighbours as well as the effects of plant order of arrival on above and belowground performance, however, have been less tested, and little is known about exactly how roots perform when they have a legume neighbour. We do not know whether roots of species that benefit from N facilitation actually grow towards legume roots to obtain extra N or whether their improved N uptake is purely a physiological process (in the sense of an improved nutrient uptake and nutrient use efficiency).

Studying root traits and interactions is a major challenge since belowground systems are hidden, and their study depends on technologies that provide access to roots and their interactions, many of which have only recently been developed (Faget *et al.* 2013a; b; Oburger & Schmidt 2016). Techniques using fluorescence markers within genetically transformed plants expressing green or red fluorescent proteins (*gfp, rfp*) have opened up crucial opportunities to follow root interactions and growth at least under controlled conditions. This makes it possible to distinguish the roots of different species sharing the same soil compartment (Faget 2009; Faget *et al.* 2010, 2012), thus opening up avenues to a better understanding of how root-root interactions contribute to competitive and facilitative outcomes and ecosystem functioning (Faget *et al.* 2013b).

Here we report the findings of a controlled rhizotron experiment in which we manipulated interactions between maize with a legume and/or non-legume neighbour. We grew maize alone, with only a legume, only a grass, or with both, and we tracked roots using green and red fluorescent markers (Faget 2009; Faget *et al.* 2010). We wanted to know how maize shoots and roots perform when growing alone or with neighbours, and if there was any difference if the neighbour is a legume (potentially a strong facilitator, but also a strong competitor) or a grass (potentially a strong competitor). We also investigated if the spatial location of the neighbours and the order of arrival would affect maize performance. Thus, we tested the effects of neighbour identity and spatial location, as well as order of arrival on maize above and belowground performance. We aimed to answer the following questions:

1. Is maize above and belowground performance affected by having a neighbour, as well as by the identity of the neighbour, its spatial location and the order they arrive (priority effects)?
2. Do maize roots tend to grow more towards the legume than towards the grass roots?

METHODS

Plant material

In order to study interactions between plants we used maize (*Zea* sp.) as focal species, and two neighbours species: wheat (*Triticum* sp.) and clover (*Trifolium pratense* L., a legume species). To be able to distinguish the roots in the soil, we used genetically transformed maize including the gene for *gfp* (Green Fluorescence Protein) in compliance with (Faget 2009; Faget *et al.* 2010, 2012); and a genetically transformed wheat including the gene for *rfp* (Red Fluorescence Protein) according Faget *et al.* (2013). Clover was grown as wild type whose roots could be distinguished from the other two species with coloured roots.

Experimental conditions and set up

The experiment was conducted in the greenhouse of the Institute of Plant Sciences (IBG-2) in the Forschungszentrum Jülich in Germany, and lasted from October to November 2013, a total of four weeks from the first sowing to harvest. Rhizotrons (29.5 cm x 59.5 cm x 3.5 cm) were filled with 5 litres of low nutrient soil (low nutrient commercial potting soil mixed with nutrient solution). We kept the soil nutrient status low so that the legume would depend mainly on atmospherically fixed N. All rhizotrons were kept in the greenhouse (average 69% humidity, average temperature 23.1°C day, and 19.6 °C night) and placed at angle of 45° forcing roots to growing along the transparent interface. Each rhizotron was watered to maintain the soil moisture at 25% volumetric water content throughout the experimental period.

We sowed maize alone or together with one or two other species, wheat and/or clover (Fig. 5.1). Seeds of clover were sown directly in the rhizotrons. Seeds of maize and wheat were sown on blotting paper for germination, in order to confirm the proper fluorescence activity when exposed to excitation light, before transplanted to the rhizotrons.

Seven treatments and five replicates per treatment (35 rhizotrons) were set up (Fig. 5.1), to verify if the identity and the spatial distribution of the neighbours, as well as the plant order of arrival would affect the above and belowground performance of maize, which was the focal individual placed in the centre. In the five treatments with a order of arrival component, the species were sown with five days difference (called non-simultaneous sowing) with the following plant distribution: maize growing alone (M), maize interacting with one species, either wheat (WW-WW) or clover (CC-CC), and maize interacting with two species placing wheat and clover at two different positions in the rhizotron around the focal maize (CW-WC, e.g. clover on the outside, wheat next to maize; and WW-CC, e.g. clover on one side, wheat on the other). In addition, two more treatments were established with the same distributions as the two last ones, but where all species were sown at the same time (sCW-WC and sWW-CC), which we called simultaneous sowing treatments. Since it was important to have similar sized plants to compare, but the three species used grow at different rates, we sowed species at different time points (non-simultaneous sowing treatments) in five of the treatments. Clover, the slowest grower was sown first, followed by wheat and then maize, with five days between each sowing. These treatments also provided a priority effect approach. The two treatments sown at the same time then became the control treatments for the question of whether priority effects would ensue if species were sown at different times.

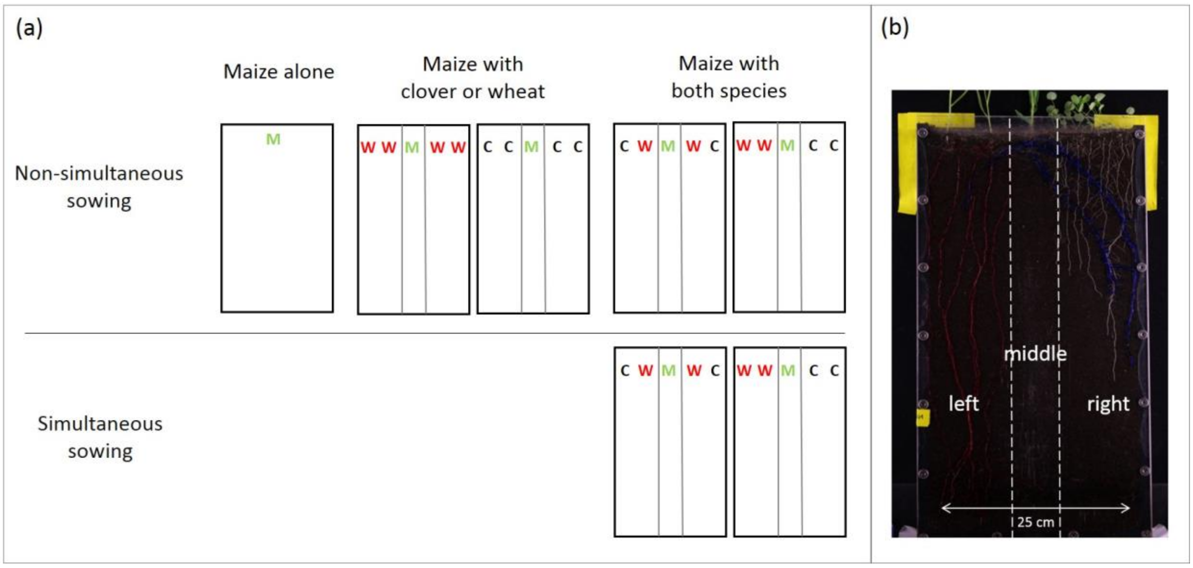


Figure 5.1. a) Experimental design representing rhizotrons and the seven treatments, where maize (M) was sown alone, or together with wheat (W) and/or clover (C), at the same or at different times. Each treatment had five replicates giving a total of 35 rhizotrons. Letter (M) represents one individual of maize, (W) one of wheat, and (C) five of clover, summing to one maize, two or four wheat, and ten or twenty clover individuals per rhizotron depending on the treatment composition. b) Each rhizotron was divided into three zones: the middle, where the focal plant (maize) was sown, and right and left, where the neighbour species were sown at different spatial locations (see Fig. 1a). The example rhizotron in the photo shows the treatment WW-CC.

We divided the rhizotron area virtually into three compartments with a central band of 5 cm in which we sowed one individual of maize (focal plant) and two sides of 10 cm each. These right and left sides were used to sow the neighbour plants: two plants of wheat or ten plants of clover per side when there was only one species on either side of the maize, or this number divided by two when two neighbours were grown together on one side of the maize. Thus, when maize interacted with one species the sowing density was twenty individuals of clover and four of wheat, whereas in the treatments where maize interacted with two species, ten individuals of clover and two of wheat were sown (Fig. 5.1a). For data analysis, we used the three vertical zones described above: the middle zone under the maize plant (5 cm width) and a left and right zones (10 cm width each) under the neighbour species where the species were distributed according each treatment (Fig. 5.1).

Sampling and screening data

In order to identify the roots from different species we used an imaging system for identifying roots that can emit green and red fluorescence from transgenic maize and wheat respectively. With proper lighting and filtering, only roots of plants expressing the *gfp* or *rfp* were visible (Faget 2009; Faget *et al.* 2010, 2013a; b), which allowed us to register each root position and trace it on each measurement day, drawing them manually on the transparent interface of the rhizotron. We were able to identify roots of maize in green (drawn in blue on the rhizotron for a better contrast), wheat in red and non-coloured roots for clover (Fig. 5.1b). After identifying and tracing the root position at the interface of the rhizotrons, they were imaged with a conventional camera system for further analysis (digital camera Canon G10 mounted on a tripod).

In order to analyse the root system architecture, the images of roots acquired with the conventional camera were analysed using the software GROWSCREEN-Root (adapted from Nagel el al. 2009). With this software we manually traced maize roots drawn before on the transparent interface of the rhizotrons, using a computer mouse graphics table with pen (Wacom Cintiq 21UX, CANCOM Deutschland GmbH, Düsseldorf, Germany). We obtained then the total root length, first order root length and lateral root length of maize per rhizotron. The GROWSCREEN-Root software allowed us to divide the root analyses in left, middle and right zones of each rhizotron, resulting in roots traits of maize for each zone and each treatment as presented above.

The images of roots were acquired three weeks after the sowing events (maize seedlings had the same age at harvest). Maize shoot of all rhizotrons was harvested and plant shoot material dried in the oven (at 70°C, 48 hours) and aboveground dry weight was measured. Soil samples were also taken in the beginning and the end of the experiment. Soil and shoot material were milled to analyse for leaf N% and soil %C, %N, %K and %P (VarioelCube Elementar and ICP-OES methods).

Statistical analyses

We analysed maize as a focal plant and the effects of seven treatments growing maize with different neighbour species and different location. We measured the effects of neighbour identity, neighbour spatial location and order of arrival on maize aboveground traits (biomass, leaf % N and leaf C/N) and belowground traits (total root length, lateral root length and first-order root length). The root length density was calculated as the ratio between the measured total root length (cm) and analysed area (cm^2), thus all the belowground results are expressed in cm/cm^2. We performed one-way ANOVAs in order to test the effects of the treatments on above and belowground traits. When the null hypothesis was rejected ($P<0.05$), the mean treatment values were compared with a Newman and Keuls test performed with the R package agricolae (de Mendiburu 2015). All statistical analyses were performed using R 3.3.1 (R Core Team 2016).

In order to compare roots distribution between the right and left rhizotron zones in each treatment we calculated an adapted version of the Relative interaction index (RII). It was calculated for each rhizotron as:

$RII = \text{root length}_{left} - \text{root length}_{right} / \text{root length}_{left} + \text{root length}_{right}$.

Treatments in which the 95% confidence interval (CI) of the RII includes negative values indicate a preference for the left side, while positive values indicate a preference for the right side, and zero indicate that roots are equally distributed between the right and left zones.

RESULTS

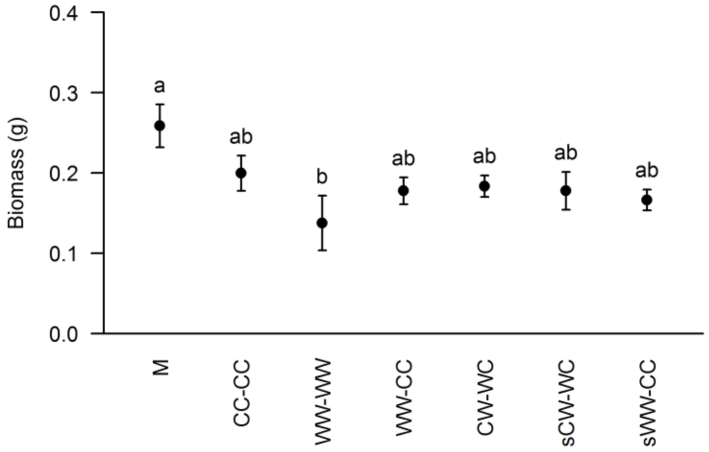
Effect of neighbour identity, spatial location and order of arrival on maize aboveground and belowground performance

Our results showed that maize aboveground biomass, maize leaf N and C/N were significantly affected ($P=0.016$; $P<0.001$; $P<0.001$) by the seven treatments which tested the effects of neighbour identity, spatial location and order of arrival (Fig. 5.2a,b,c). The statistically significant difference found in aboveground biomass was due to the difference between maize growing alone and maize grown with wheat on both sides, while no differences were found between the treatments where maize was grown alone or with clover as neighbour, no matter the spatial location (Fig. 5.2a). Thus, the spatial location of the neighbours as well as the order of arrival did not affect maize aboveground biomass.

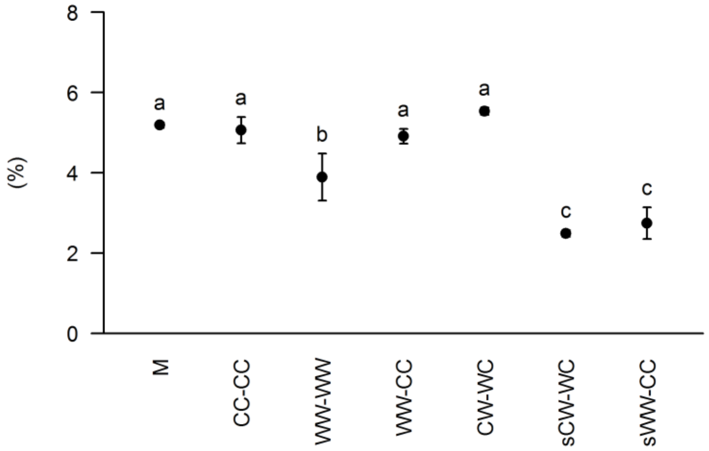
Maize leaf N in the treatment where maize was grown with wheat in both sides (WW-WW) was lower than when growing alone and in all the non-simultaneous sowing treatments (Fig. 5.2b). The simultaneous sowing treatments were significantly different from all the non-simultaneous treatments. Consequently, the exact opposite pattern was found in maize leaf C/N (Fig. 5.2c).

With regard to maize total, lateral and first-order root length in the whole rhizotron, there were no statistically significant differences between the treatments (Fig. 5.3a,b,c).

(a) Maize aboveground biomass



(b) Maize leaf N



(c) Maize leaf C/N

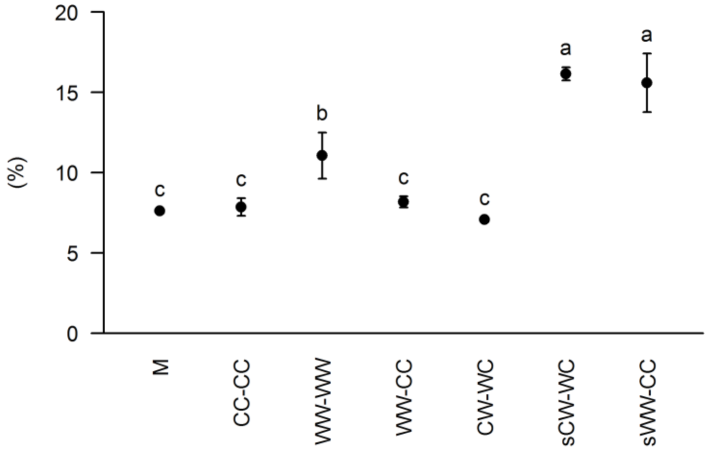


Figure 5.2. Influence of neighbour identity (whether clover or wheat), order of arrival and spatial location on maize aboveground biomass, leaf %N and C/N. Treatments names are explained in Fig. 1A. The values are means plus/minus one standard error of the mean.

Different letters show significant differences between treatments (ANOVA followed by a Newman and Keuls test, $P < 0.05$).

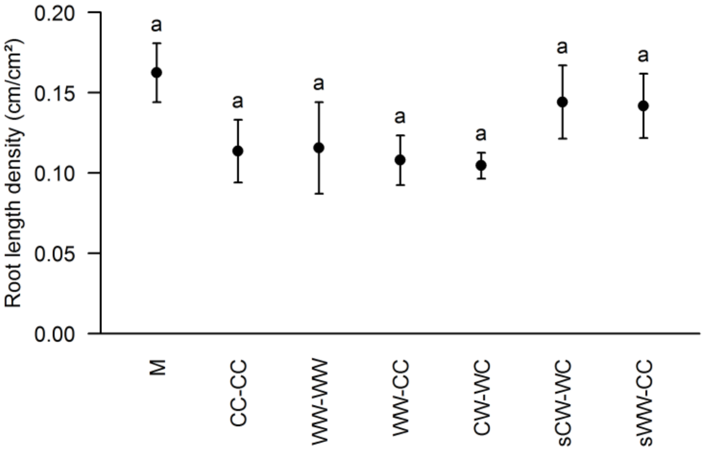
Maize root distribution in the rhizotron zones

Looking to the roots distribution between the right and left rhizotron zones of the rhizotrons, we found in the WW-CC treatment a preference of maize total and lateral root length to grow in the right side, where clover was sown, as indicated by the positive values of RII (Fig. 5.4a,b). In the CW-WC treatment maize total and lateral root length tended to grow in the left side, as indicated by the negative values of RII (Fig. 5.4b). Treatments in which the 95% CI include zero, indicate that any preference was found (Fig. 5.4a,b,c).

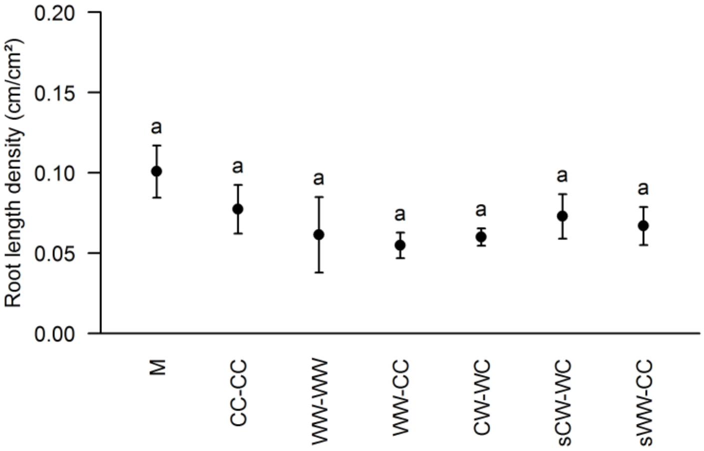
Soil chemistry

We provide data on soil chemical parameters measured before and at the end of the experiment in Table 5.1 of Supporting Information. No significant differences of soil %C, %N and %P were found between the seven treatments, whereas the sWW-CC treatment had the lowest %K values. Generally, there was no significant different in soil nutrients between the start and the end of the experiments, except for a significantly decrease in soil % N and % P, and an increase of % K.

(a) Maize total root length



(b) Maize lateral root length



(c) Maize first-order root length

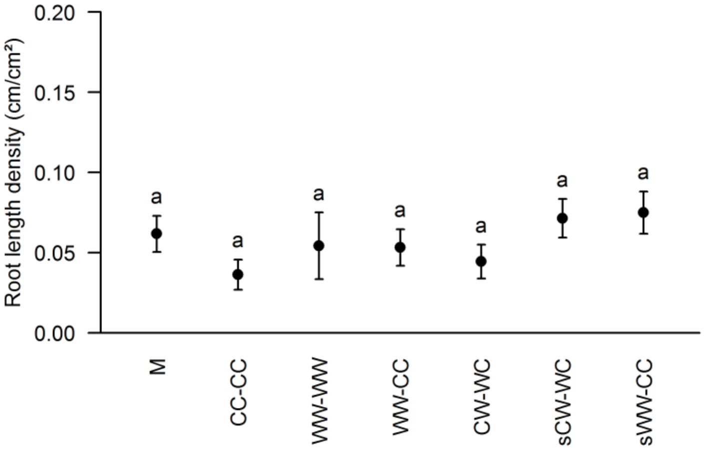


Figure 5.3. Influence of neighbour identity (whether clover or wheat), order of arrival and spatial location on maize total, lateral and first-order root length density in each. Treatments names are explained in Fig. 1A. The values are means plus/minus one standard

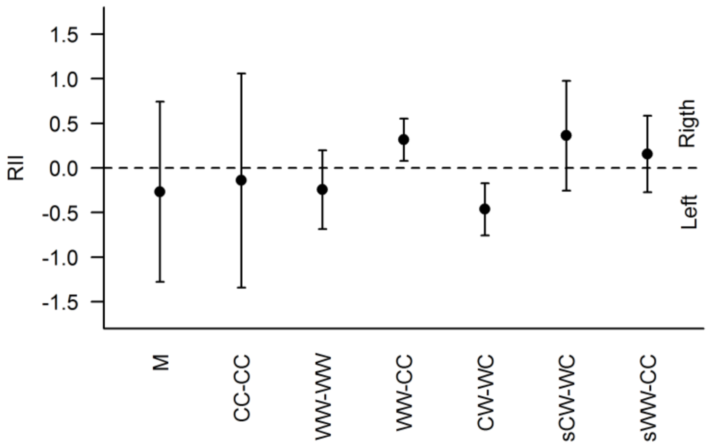
error of the mean. Different letters show significant differences between treatments (ANOVA followed by a Newman and Keuls test, $P < 0.05$).

DISCUSSION

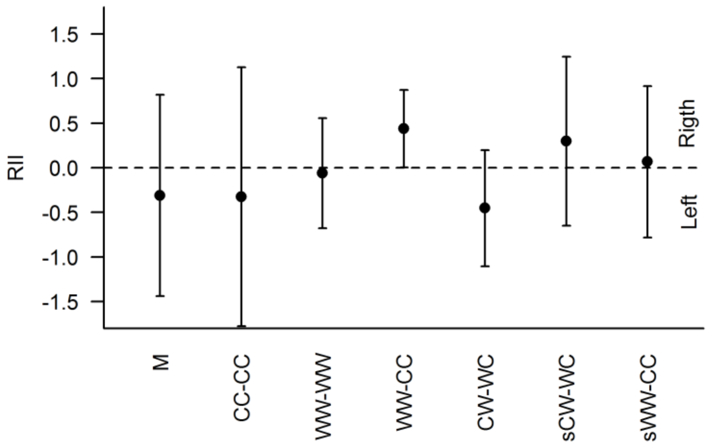
In answer to our first question, maize aboveground was affected by having a neighbour, as well as by the identity of the neighbour, its spatial location and the order they arrived. However, maize roots did not respond to the treatments when looking to the entire rhizotrons.

Maize aboveground biomass was higher when it was sown alone or with clover than when grown together with wheat. Thus, maize performed similarly when growing alone or with a legume, but differently when growing with wheat, indicating that different effects are found depending on the identity of the neighbour (mainly from which functional group the neighbour belongs to). The species-specific effect we found is likely to be a functional trait effect driven by the typical traits of the functional group the neighbour belonged to, in this case legumes, with their ability to fix atmospheric N. However, since we only tested one legume and one grass neighbour we cannot entirely confirm this pivotal seeming role of functional group identity here.

(a) Maize total root length



(b) Maize lateral root length



(c) Maize first-order root length

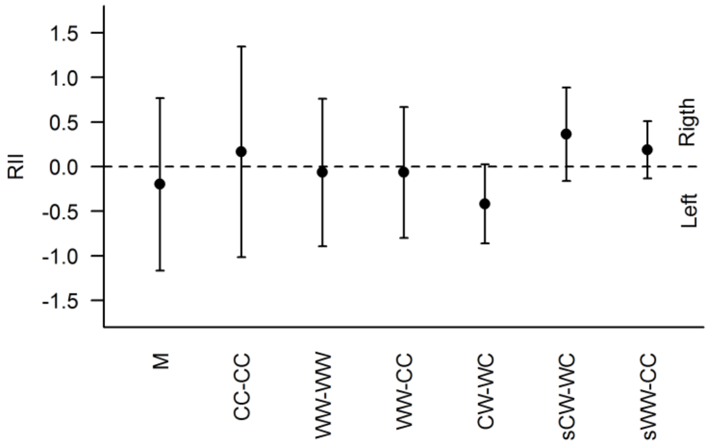


Figure 5.4. Roots distribution between the right and left rhizotron zones in each treatment expressed by an adapted version of the Relative interaction index (RII). Mean values are shown with the arrows indicating the 95% confidence interval range. Negative values show a preference for the left side, while positive values for the right.

Maize leaf N and C/N data showed evidence for N facilitation since leaf N in all the non-simultaneous sowing treatments containing a clover was higher than the treatment containing only wheat. In addition simultaneous and non-simultaneous sowing treatments resulted in significantly different maize leaf N and C/N. The simultaneous sowing treatments had the lowest maize leaf N% values and the highest C/N. This suggests that when maize was sown at the same time as the neighbours (instead of after for the other two species) either it was not able to take up soil N (which would be surprising, since one would expect it to perform better when arriving at the same time rather than after the other species) or it benefitted from the legumes sown ten days before maize arrived in the non-simultaneous treatments. The latter would be evidence for N facilitation, which backs up the overall leaf C/N data. Field and controlled experiments testing plant order of arrival (priority effects) showed that sowing legumes before other plant functional groups results in higher aboveground biomass and productivity (Körner *et al.* 2008; von Gillhaussen *et al.* 2014; Weidlich *et al.* 2017). They hypothesize that species sown after legumes may have a better chance of establishing as a result of their smaller root systems, providing more root foraging opportunities for the other species, as well as a possible role of N sparing and/or extra N transfer of nitrogen fixed by legumes to subsequent neighbours.

We also measured the δ15N signal in leaves but found (as had occurred in previous studies) that the natural abundance method for assessing whether N coming from a legume source was present in a neighbour benefitting from the legume presence (Shearer & Kohl 1986) does not work in rhizotrons. Values of δ15N obtained in rhizotrons unfortunately do not reflect the typical N fixation signals (between -2 and 0) for legumes nor the values typically found for neighbours near a legume. For this reason we do not report δ15N data in this study. Many studies have shown positive N facilitation effects of the vicinity of legumes on leaf N of neighbours, such as in intercropping settings (Li *et al.* 2003) or in grasslands (Spehn *et al.* 2002; Temperton *et al.* 2007; Brooker *et al.* 2008). Since our study was a short-term experiment, this probably played a role in the lack of stronger evidence for N facilitation in maize.

Our results showed that competition was stronger in the treatments where maize had only wheat as a neighbour, but as soon as the treatment was also composed of clover, maize could develop better, indicating a form of facilitation effect of the legume species. This result suggests that direct facilitation may have being playing a role in the CC-CC and WW-CC treatments, while indirect facilitation may have occurred in the CW-WC treatments (with clover located on the outside of the wheat), where maize benefited from the effects of clover on wheat even having wheat as the closest neighbour. The soil chemistry data (Table 5.1 in Supporting Information) shows clearly that there were very minor changes in concentrations of the main elements in the soils between the start and the end of the experiment. Equally soil N did not increase in the treatment with only clover as a neighbour for maize. This suggests that facilitative interactions may play off in a sequence of different adaptations, including initial changes in root foraging towards legumes followed by improved N uptake over time (which would have possible been seen also increased leaf N in a longer duration experiment, as well as more soil N availability). In contrast with studies which showed an increase of P uptake when intercropping with a legume (Li *et al.* 2003; Hinsinger *et al.* 2011), soil P did not increase along the experiment and was not different between the treatments. Testing the effects of intercropping systems (maize, faba bean and wheat), Fan et al. (2006) found higher yield when maize intercropped with faba bean (a legume) than with wheat. Temperton et al. (2007) postulated that competition for soil nitrate was reduced in plots containing legumes since they did not need to take up much soil N, leaving the spare N for the neighbours. Bessler et al. (2012) also found higher N uptake in grasslands with legumes, and Felten et al. (2016) found positive effects of legumes on N concentration suggesting that N sparing plays a role for species N uptake.

We could positively answer our second question since when looking to the roots distribution between the right and left rhizotron zones of the rhizotrons, maize roots grew more towards the legume than towards the wheat neighbour. It shows that the identity of the neighbour as well as its spatial location played a role in roots distribution. We found in the treatment where wheat was in one side and clover in the other (WW-CC) a preference of maize total and lateral root length to grow in the right side, exactly where the legume was sown. Thus, the total amount of roots was not affected by the treatments, but what varied was the root system architectural traits depending on the neighbour's identity. This points towards local adaptations of root foraging depending on who and exactly where the neighbour was. The RII results shows clearly that when wheat was sown in one side and clover was sown in the other side, maize total and later root length were higher in the zone occupied by the legume. This also confirms the statement that as soon a clover is present in the association, maize roots can develop better by being less affected by wheat competition, and that maize tended to grow more towards the legumes rather than the grass.

The simultaneous and non-simultaneous sowing treatments did not affect maize roots distribution in the left and right zones. This is possibly the result of lower competition in the simultaneous sowing when comparing with the no-simultaneous, where the first species sown developed roots before the others. If a similar experiment would least for a longer time, competition may also increase in the simultaneous sowing treatments, but due to time limitation because of the rhizotron size constraints, we could not evidence this effect as strongly as the others.

Overall, we found evidence that maize development was negatively affected by having a grass neighbour, while it did as well as with a legume neighbour as alone. In addition, as far as we know, we showed for the first time that more roots develop close to legume neighbours compared to grass neighbours. This is what we were expecting to find in terms of root foraging behavioural adaptation to having a legume neighbour that brings more N into the system. Thus, we can infer from our study that having legume as a neighbour is an advantage due to direct and/or indirect facilitation in a mixture with other competitor species. Our findings that the priority effects treatments affected the outcome in a spatially explicit way (aboveground) show that the timing of arrival also affects local adaptations of a non-legume species to its neighbourhood. Whilst we did not test density effects of sowing/planting different densities of individuals per species, we did not expect that any density effects would be larger than neighbour identity or exact spatial location effects. We are aware that controlled experiments, such as the one performed in this study, limits to bring a more ecosystem-level understanding, however, it allowed a better mechanistic understanding on how legumes interacted with non-legumes.

These results are important within the context of gaining a better understanding of the interplay of competition and facilitation (Brooker *et al.* 2008). They also extend our knowledge of facilitative effects between legumes and their neighbours to include spatial location, and show that non-legume roots actually grow towards the legume (even if we yet know nothing about possible improved physiological N uptake mechanisms that may also play a role in facilitation). Such findings have implications for intercropping in agricultural systems (Hauggaard-Nielsen *et al.* 2008; Postma & Lynch 2012) or within biodiversity-ecosystem functioning settings in more species-rich habitats. We conclude that the identity of the neighbours, together with their spatial location plays a key role in plant-plant interactions, and that the order the species arrive in the system can modulate the outcome of these interactions.

SUPPORTING INFORMATION CHAPTER 5

Supporting Information 5.1. Soil chemistry for total carbon, nitrogen potassium and phosphorus in the seven different experimental levels of the factor neighbourhood at the beginning of the experiment and at harvest time at the end of the experiment. Values are means plus/minus one standard deviation.

Treatment	C %	N %	K mg/kg	P mg/kg
Time zero	25.81 ± 1.65	0.50 ± 0.03	5210.00 ± 640.51	775.00 ± 37.75
M	22.99 ± 2.97	0.46 ± 0.06	6269.78 ± 758.85	672.07 ± 67.88
WW-WW	23.28 ± 1.46	0.44 ± 0.03	6173.10 ± 500.27	676.04 ± 33.91
CC-CC	23.13 ± 1.87	0.43 ± 0.04	6185.82 ± 642.34	669.50 ± 57.94
CW-WC	23.96 ± 1.87	0.45 ± 0.05	6155.16 ± 548.92	697.39 ± 74.13
WW-CC	23.55 ± 2.01	0.44 ± 0.05	6096.96 ± 651.67	673.04 ± 67.72
sCW-WC	23.95 ± 1.62	0.46 ± 0.04	5741.30 ± 599.69	644.35 ± 37.51
sWW-CC	24.04 ± 2.03	0.47 ± 0.05	5422.08 ± 615.03	677.50 ± 57.39

CHAPTER 6

Paper accepted in March 2017

Accuracy and bias of methods used for root length measurements in functional root research

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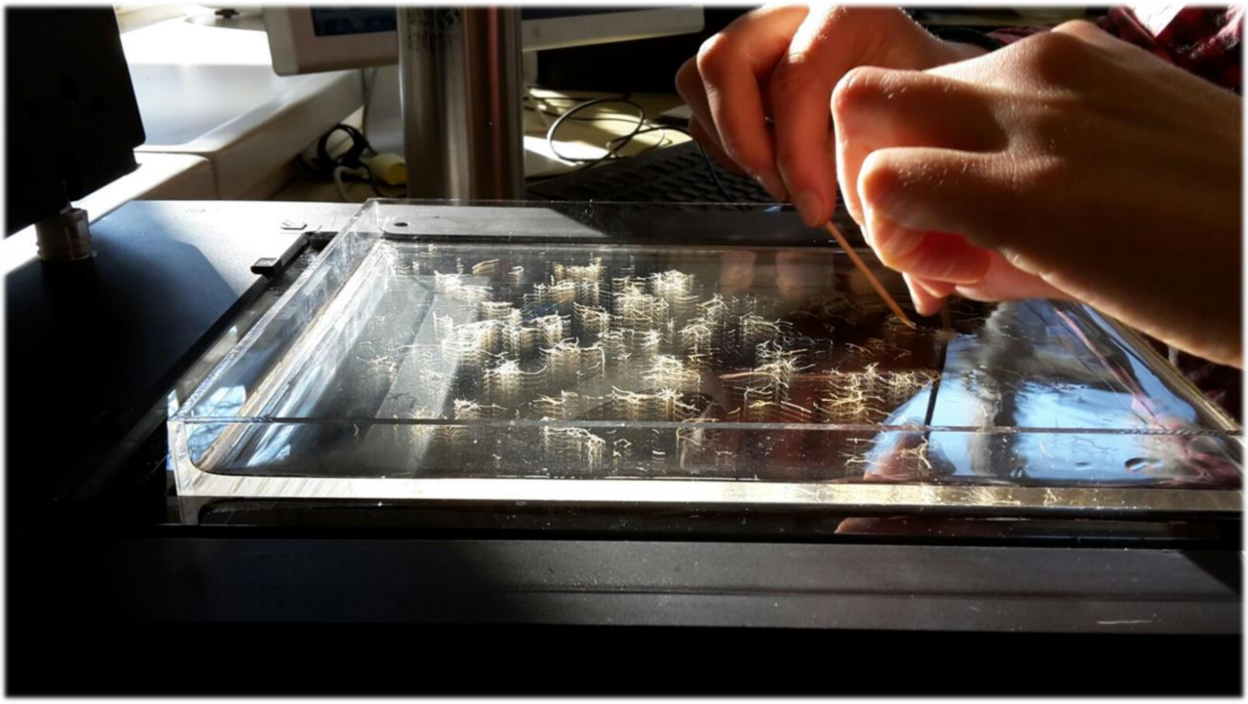
BM Delory, EWA Weidlich, L Meder, A Lütje, R van Duijnen, R Weidlich, VM Temperton (2017). Methods in Ecology and Evolution.



ABSTRACT

1. Functional root traits are becoming a key measure in plant ecology, and root length measurements are needed for the calculation of root traits. Several methods are used to estimate the total root length (TRL) of washed root samples (e.g., modified line intersect [LI] method, WinRHIZO^{TM}, and IJ_Rhizo), but no standardised comparison of methods exists.
2. We used a set of digital images of unstained root samples to compare measurements given by the LI method and automated methods provided by WinRHIZO^{TM} and IJ_Rhizo. Linear regression models were used to detect bias. Both linear regression models and the Bland-Altman`s method of differences were used to evaluate the accuracy of eight methods (1 manual, 2 semi-automated, and 5 automated) in comparison with a reference method that avoided root detection errors.
3. Length measurements were highly correlated but did not exactly agree with each other in 11 out of 12 method comparisons. All tested methods tended to underestimate the TRL of unstained root samples. The accuracy of WinRHIZO^{TM} was influenced by the thresholding method and the root length density (RLD) in the pictures. For the other methods, no linear relationship was found between accuracy and RLD. With WinRHIZO^{TM} (global thresholding + pixel reclassification; RLD = 1 cm cm^{-2}), the Regent's method and the Tennant's method underestimated the TRL by 7.0 ± 6.2% and 4.7 ± 7.9%, respectively. The LI method gave satisfactory results on average (underestimation: 4.2 ± 6.0%) but our results suggest that it can lead to inaccurate estimations for single images. In IJ_Rhizo, the Kimura method was the best and underestimated the TRL by 5.4 ± 6.1%.
4. Our results showed that care must be taken when comparing measurements acquired with different methods because they can lead to different results. When acquiring root images, we advise to (1) increase the contrast between fine roots and background by staining the roots, and (2) avoid overlapping roots by not exceeding a RLD of 1 cm cm^{-2}. Under these conditions, good length estimates can be obtained with WinRHIZO^{TM} (global thresholding + pixel reclassification). The Kimura method in IJ_Rhizo can be an alternative to WinRHIZO^{TM}.

INTRODUCTION

Often referred to as ‘the hidden half’ (Eshel & Beeckman 2013), plant roots serve multiple functions simultaneously and are an important driver of ecosystem processes (Gregory 2006; Bardgett, Mommer & De Vries 2014). Root systems provide anchorage, a network for water absorption and nutrient uptake, and alter the physicochemical properties of the rhizosphere via the exudation of a great diversity of low and high molecular weight metabolites into the soil (Delory *et al.* 2016; Mommer, Kirkegaard & van Ruijven 2016; Rellán-Álvarez, Lobet & Dinneny 2016). In addition, the rhizosphere houses a number of organisms developing interactions with roots that can have positive or negative effects on plant health (Hinsinger *et al.* 2009; Raaijmakers *et al.* 2009).

Although plant roots play a central role in ecosystem functioning (Bardgett *et al.* 2014), plant scientists and ecologists face many technical challenges in measuring root traits. There are two main reasons for this: (1) the belowground location of roots that hampers direct observations (Pagès *et al.* 2010), and (2) the impossibility of species identification based on simple root morphological markers in species-rich plant communities (see Mommer *et al.* 2008, 2010, 2011; Faget *et al.* 2013 for methods to identify roots of different species). Among all the possible root traits that can be measured on root samples extracted from soil cores, the specific root length (SRL, the root length per unit root biomass) and the root length density (RLD, the root length per unit volume of soil) are of particular interest (Bardgett *et al.* 2014; Kramer-Walter *et al.* 2016; Weemstra *et al.* 2016). While SRL is a morphological trait that provides information about the amount of resources needed to increase the surface area between roots and soil (Kramer-Walter *et al.* 2016), RLD is an architectural trait describing the capacity of a root network to explore a given volume of soil and acquire limited resources (Hecht *et al.* 2016; Ravenek *et al.* 2016). As a prerequisite for calculating SRL and RLD, one has to know the total root length (TRL) in the studied samples.

The first method developed to compute the TRL from washed root samples was presented by Newman in 1966. In his paper, Newman (1966) derived an equation estimating the TRL in a sample by counting the number of intersections between randomly orientated roots evenly distributed in a tray of known area and randomly oriented straight lines of known total length. A few years later, Marsh (1971) and Tennant (1975) simplified Newman’s equation by using a grid of regularly spaced lines crossing the roots. This line intersect method became rapidly popular among plant scientists and ecologists because it is easy to use, not expensive, and faster than any other manual length measurement method (Tennant 1975). The rapid development of computer hardware and software led scientists to develop image analysis algorithms able to compute the TRL from captured images of washed root samples (Ewing & Kaspar 1995; Kimura, Kikuchi & Yamasaki 1999; Pierret *et al.* 2013) and analyse 2D root system architectures (see the Plant Image Analysis Database described in Lobet *et al.* 2013).

Different image analysis methods rely on a different set of assumptions and care must be taken when choosing the best software package to analyse a set of digital images (Pridmore, French & Pound 2012). Next to the line intersect method, two software packages can be used to easily compute the TRL from scanned root samples: the commercial software package WinRHIZO^{TM} (examples of studies using WinRHIZO^{TM}: Mommer *et al.* 2012; Pagès & Picon-Cochard 2014) and the open-source ImageJ macro IJ_Rhizo (Pierret *et al.* 2013). Even if previous studies showed that a strong linear relationship exists between measurements acquired with WinRHIZO^{TM} and IJ_Rhizo (Wang & Zhang 2009; Pierret *et al.* 2013), the agreement between the methods provided by these software packages and the manual line intersect method has not yet been investigated. In addition, the accuracy of these methods is poorly known and has been reported to be strongly related to the settings used for image acquisition and analysis (Bouma, Nielsen & Koutstaal 2000). Previous studies reported that differences between reference values and measurements acquired with WinRHIZO^{TM} (Himmelbauer, Loiskandl & Kastanek 2004) or the line intersect method (Goubran & Richards 1979) did not exceed 5% when the RLD on the scanning area was low (around 1 cm cm^{-2}).

Given the increasing number of studies dealing with root trait measurements and considering that different methods can lead to different results, we performed a comparative study of several methods commonly used in functional root research to estimate the TRL of washed root samples. We wanted to see if results computed with different methods can be safely compared or if different methods led to significantly different absolute measurements of the same quantity. We also aimed to assess to what extent researchers without access to expensive software and equipment may be able to rely on freely-available software solutions (such as ImageJ and IJ_Rhizo). In this paper, we used a set of 50 digital images of unstained roots collected from soil cores during a grassland field experiment (Jülich, Germany) to compare the length estimates given by the manual line intersect method (Tennant 1975) and the automated methods provided by WinRHIZO^{TM} and IJ_Rhizo (Pierret *et al.* 2013). We designed this study in order to answer the following questions:

1. Do the tested methods agree with each other?
2. Are the tested methods accurate?

MATERIALS AND METHODS

Root images

The roots analysed in this study were collected from soil cores in 2014 in a grassland experiment located in Jülich (Germany). After collection, the roots were carefully washed and stored in a freezer (-20 °C). Before image acquisition, the roots were gently defrosted and spread in a thin layer of water in a transparent plastic tray. The roots were cut into small segments (1-3 cm) in order to facilitate spreading and minimise overlapping. We did not use a dye to stain the roots prior to image acquisition because such an operation is not always possible, particularly when additional analyses/measurements have to be performed on roots after scanning (e.g. chemical analyses). For each sample, one grey level image and one colour image were acquired with a flatbed scanner (Epson Perfection V800 Photo). First, the roots were scanned in transparent mode at a resolution of 400 dpi. The root density in the tray was found to be between 0.1 and 2.9 cm cm^{-2}. The scans were then saved as 8 bits per pixel grey level images. Then, the same roots were scanned in colour at a resolution of 800 dpi using the scanner’s reflective lighting system. A white background with a green 2×2 cm grid was placed behind the roots before the second scanning. These scans were saved as 24 bits per pixel colour images.

Root length measurements

In order to evaluate the agreement between popular root length measurement methods, 50 images were randomly selected and analysed using one manual and five automated methods developed to estimate the TRL from washed root samples (Fig. 1). The grey level images were analysed with the commercial software package WinRHIZO^{TM} and the free ImageJ macro IJ_Rhizo. The colour images were analysed manually using the modified line intersect method according to Tennant`s guidelines (Tennant 1975).

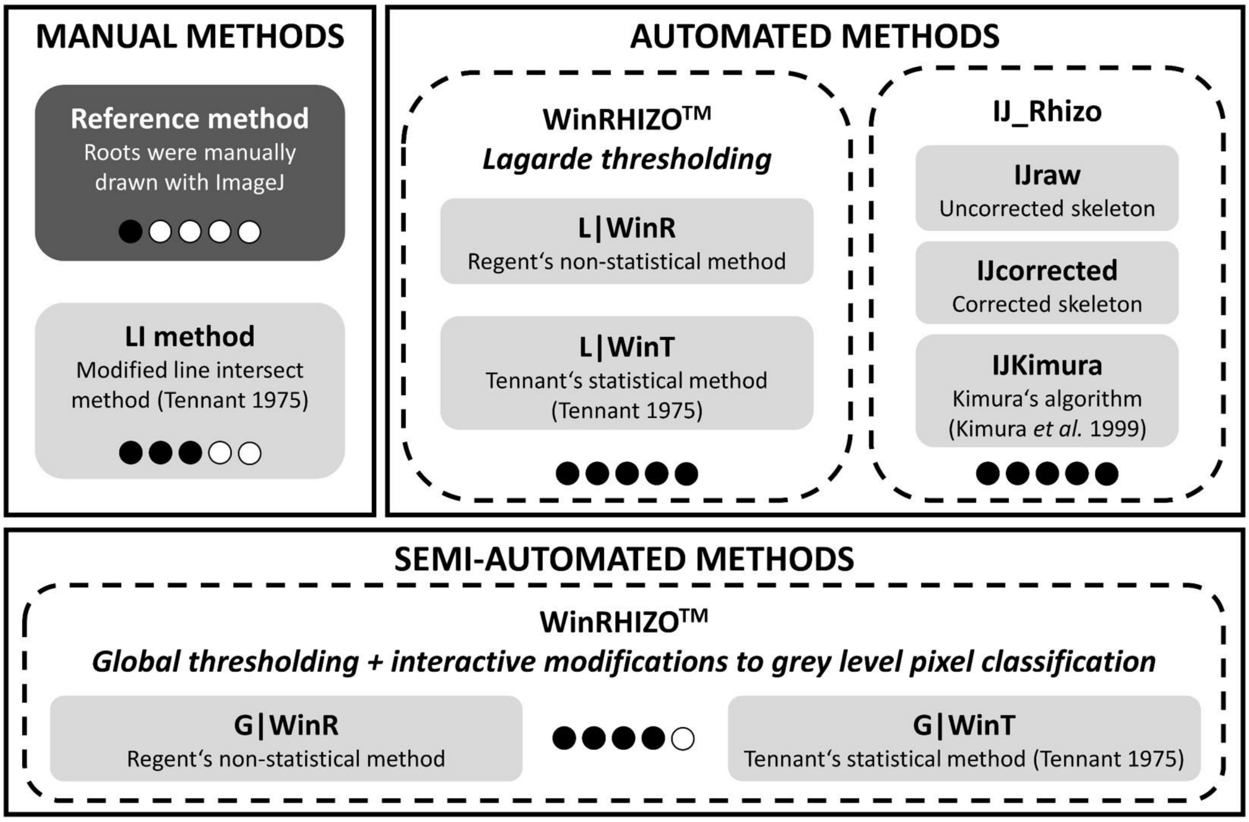


Figure 6.1. Manual, semi-automated and automated methods used in this paper for estimating the TRL of washed root samples. The methods tested in this paper are written in black (light grey background). The figure provides a qualitative assessment of the speed of each method to compute the TRL of 15 images (RLD_{min} = 0.1 cm cm^{-2}, RLD_{max} = 2.7 cm cm^{-2}, RLD_{mean} = 1.1 cm cm^{-2}, RLD_{SD} = 0.7 cm cm^{-2}). Depending on the method, the analysis of 15 images can be fast (5 black dots, less than one hour), slow (3 to 4 black dots, several hours), or very slow (1 black dot, several days).

Using WinRHIZO^{TM} Pro 2017a Pre-Release (Regent Instruments, Québec, Canada), the roots were separated from the background using a local thresholding method (Lagarde). This method was advised by Bouma *et al.* (2000) when unstained roots have to be skeletonized with WinRHIZO^{TM}. Then, TRL was computed with two different methods: the non-statistical Regent's method (L|WinR) and the Tennant's statistical method (L|WinT).

The grey level images were then also analysed with IJ_Rhizo (Pierret *et al.* 2013). Batch analyses of root images were performed with the following parameters: excluded border of 40, 50 or 60 pixels width; perform particle cleaning (size of the smallest particle: 1 mm^2; circularity of particles: 0.75); automatic thresholding. IJ_Rhizo computed the TRL derived from either the uncorrected skeleton (IJraw), the corrected skeleton (IJcorrected), or the Kimura skeleton (IJKimura). Briefly, IJraw uses the skeleton obtained after skeletonization of a thresholded image in ImageJ to estimate the TRL. Because this process removes more pixels from around thick roots than thin roots, IJcorrected adds a number of pixels to the uncorrected skeleton before estimating the TRL (Pierret *et al.* 2013). With IJKimura, pixels are discriminated and the TRL is estimated based on the number of diagonally and orthogonally connected pairs of pixels in a skeleton (Kimura *et al.* 1999).

The colour images were analysed manually using the line intersect (LI) method modified by Tennant (1975) (Fig. 6.1). With the manual LI method, the TRL (cm) was estimated following Eqn 1, where N is the number of intercepts between the roots and the grid, and D is the distance between two parallel lines of the grid (cm) (Rowse & Phillips 1974; Tennant 1975). Following Tennant's guidelines (1975), we used three different grid sizes depending on the TRL in each image: 0.5×0.5 cm (TRL < 75 cm), 1×1 cm (TRL between 75 and 275 cm), or 2×2 cm (TRL > 275 cm). When a 2×2 cm grid was used, we counted the number of intercepts between the roots and the grid using the colour images. When the LI method required a lower grid size, we superimposed a 0.5×0.5 cm or a 1×1 cm grid on the grey level images using the Grid plugin (Wayne Rasband 2007) in ImageJ 1.50b (Schneider, Rasband & Eliceiri 2012).

$$TRL = \frac{\pi}{4} \times N \times D \qquad \text{Eqn 1}$$

Because we also wanted to evaluate the accuracy of each tested method (i.e., the closeness of the outcome to an absolute and accurate value), we randomly sampled 15 pictures from the 50 images selected at the beginning of this study and we determined the TRL in each with a reference method (Fig. 6.1). To do so, all the roots were manually drawn with ImageJ and the TRL was calculated as the sum of the length of each individual segmented line (= reference method). Although time consuming, this method allowed us to avoid root detection errors and to have accurate absolute values for TRL with which the absolute values given by the methods tested in this paper can be compared. In addition, we also wanted to test if the accuracy of WinRHIZO^{TM} was influenced by the thresholding method used for root skeletonization. To do so, grey level pixel classification within the 15 images selected previously was performed with a Lagarde's local threshold (L) or with a global threshold (G). With the latter, a single threshold value was chosen automatically to classify all pixels of an analysed region. Then, this value was adjusted manually for some parts of the images following the procedure described in the Regent's technical support manual in order to avoid missing roots. With both thresholding methods, the TRL was computed with the Regent's method (L|WinR or G|WinR) and the Tennant's method (L|WinT or G|WinT). Therefore, the accuracy of one manual, two semi-automated, and five automated methods was evaluated in this study (Fig. 1).

Data analysis

First, we performed pairwise comparisons between six tested methods (WinRHIZO^{TM} with Lagarde thresholding vs LI or IJ_Rhizo; LI vs IJ_Rhizo) (Table 6.1, Fig. 6.2). In addition, the values obtained with each method were compared to the values calculated with the reference method (Table 6.2, Fig. 6.3). When two methods were compared, the strength of the linear relationship between the TRL estimates was assessed by calculating the Pearson's product-moment correlation coefficient and performing a Model II linear regression (ordinary least products). We chose this type of regression model because the *x* values were not fixed by the experimental design and were thus subject to error (Ludbrook 1997, 2010a; Legendre & Legendre 1998). The regression models were fitted using the R package lmodel2 (Legendre 2014) and were used to search for fixed and proportional bias. We considered that a method had a fixed bias if it gave values that were consistently higher (or lower) than a second method. In contrast, if the difference between length estimates given by two methods increased (or decreased) with the TRL in a picture (Ludbrook 1997), we considered this a proportional bias. For each fitted model, we calculated the 95% confidence interval

(95% CI) of the slope and the intercept. Then, we tested if the regression line was significantly different from the line of equal outcomes (y=x). If the 95% CI for the intercept did not include zero, there was a fixed bias. If the 95% CI for the slope did not include 1.0, there was proportional bias (Ludbrook 1997, 2002, 2010a).

Second, we evaluated the agreement between the tested methods and the reference using the Bland and Altman's method of differences (Altman & Bland 1983; Bland & Altman 1986, 1999; Giavarina 2015) (Table 6.3, Fig. 6.4). The lack of agreement between each tested method and the reference was evaluated by calculating the 95% confidence limits (or limits of agreement). In this paper, we considered that two methods agreed with each other if the following criteria were met simultaneously: (1) no bias could be detected and (2) the calculated limits of agreement were narrow. To do so, we followed the guidelines published by John Ludbrook (2010a). We started by plotting the absolute differences between the root length estimates given by a tested method and the reference (y axis) against the average lengths given by the two methods (x axis). The strength of the linear relationship between the absolute differences and the averages was evaluated by calculating the Pearson's product-moment correlation coefficient. We also performed Model I regression analyses (ordinary least squares) in order to estimate the regression coefficients of the best linear model linking the differences between root length estimates (y) and the average values (x). Because we were only interested in predicting y from x (and not x from y), Model I regression analysis can be used (Ludbrook 2010a; b). Ordinary least squares regression models were fitted using the lm function of R (R Core Team 2016). If the correlation coefficient and the slope of the fitted model were significantly different from zero, we considered that there was proportional bias. If there was no proportional bias, the limits of agreement were calculated following Eqn 2, where $\bar{d}$ is the mean difference between a tested method and the reference, n is the number of observations, t is the quantile of the Student's t distribution (α = 0.05 and n-1 degrees of freedom), and s_{diff} is the sample standard deviation for the differences (Ludbrook 2010b). If there was a proportional bias, we constructed hyperbolic 95% confidence limits (prediction interval) around the fitted regression line using Eqn 3 (Altman & Gardner 2000), where y_{fit} is a predicted value of y for a fixed value of x, t is the quantile of the Student's t distribution (α = 0.05 and n-2 degrees of freedom), s_{res} is the residual standard deviation of y about the regression line, $\bar{x}$ and s_x are the mean value and the standard deviation of *x*, and *n* is the sample size. All statistical analyses were performed with R 3.3.0 (R Core Team 2016).

$$\bar{d} \pm \left(t_{1-\frac{\alpha}{2}} \times s_{diff} \times \sqrt{1 + \frac{1}{n}} \right) \qquad \text{Eqn 2}$$

$$y_{fit} \pm \left(t_{1-\frac{\alpha}{2}} \times s_{res} \times \sqrt{1 + \frac{1}{n} + \frac{(x - \bar{x})^2}{(n-1)s_x^2}} \right) \qquad \text{Eqn 3}$$

RESULTS

Pairwise comparisons of six methods and detection of fixed and proportional bias

Overall, we found a strong positive correlation between root length measurements acquired with the six different methods (Fig. 6.2 and Table 6.1). The correlation coefficients ranged from 0.985 (LI vs. IJ_Rhizo methods) to 0.999 (L|WinR vs. L|WinT). Our results showed that L|WinT gave on average greater length estimates than L|WinR (Table 6.1, Fig. 6.2A). When the RLD increased, we found that the manual LI method (Fig. 6.2B) and IJKimura (Fig. 6.2E) tended to give higher length estimates than L|WinR. In addition, IJraw and IJcorrected gave lower values than L|WinR (Fig. 6.2C-D), L|WinT (Fig. 6.2G-H) and the manual LI method (Fig. 6.2J-K). When comparing L|WinR and L|WinT, both fixed and proportional bias were detected (Table 6.1, Fig. 6.2A). These two types of bias were also found when the manual LI method, IJraw and IJcorrected were compared to L|WinR and L|WinT (Table 6.1, Fig. 6.2B-D and 6.2F-H), as well as when root length estimates given by IJKimura were plotted against the estimates computed by L|WinT (Fig. 6.2I). We detected a proportional bias when the following methods were compared: L|WinR and IJKimura (Fig. 6.2E), LI and IJraw (Fig. 6.2J), and LI and IJcorrected (Fig. 6.2K). No bias was detected when the length estimates given by IJKimura were plotted against the estimates given by the manual LI method, meaning that the regression line was not significantly different from the line of equal outcomes (Fig. 6.2L).

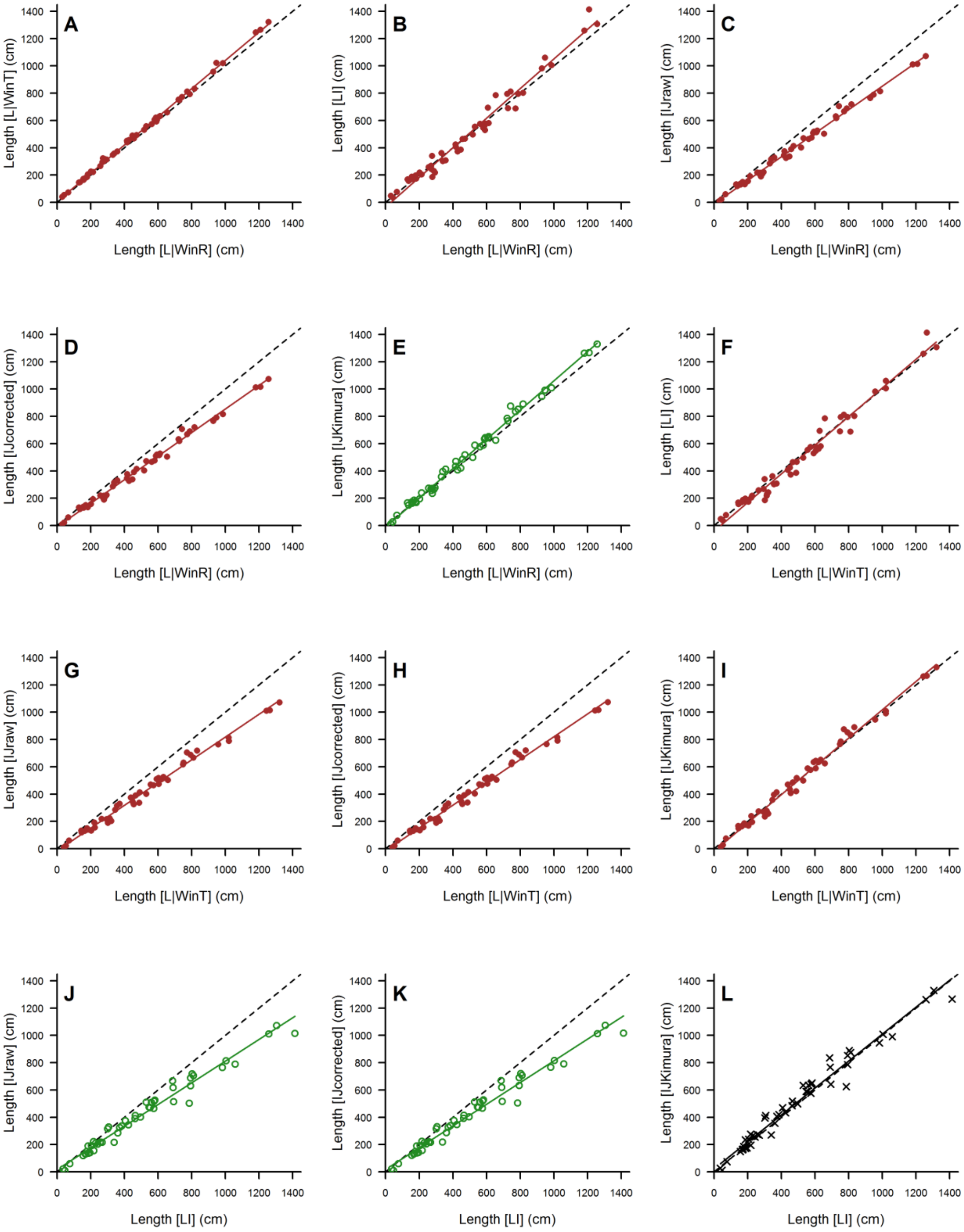


Figure 6.2. Pairwise comparisons of methods commonly used for estimating the TRL of washed root samples. On each graph, both the ordinary least products regression line (solid line) and the line of equal outcomes (dashed line) were plotted. Filled brown dots, both fixed and proportional bias; open green dots, only proportional bias; black crosses, no bias. Abbreviations are explained in Fig. 1.

Table 6.1. Pairwise comparisons of methods commonly used for estimating the TRL of washed root samples. All correlation coefficients were significantly different from zero ($P<0.001$).The 95% confidence intervals (CIs) of the regression coefficients (ordinary least products) are shown in parentheses (lower limit, upper limit) and were used to detect bias. If the 95% CI for the intercept did not include zero, there was fixed bias. If the 95% CI for the slope did not include 1.0, there was proportional bias. Method abbreviations are explained in Fig. 6.1.

Methods		Correlation (Pearson)	Linear regression		Bias		Figure
x	*y*		Slope (95% CI)	Intercept (95% CI)	Fixed	Proportional	
L|WinR	L|WinT	0.999	1.03 (1.02, 1.05)	6.12 (0.37, 11.79)	Yes	Yes	6.2A
	LI	0.988	1.09 (1.04, 1.13)	-34.72 (-58.50, -11.97)	Yes	Yes	6.2B
	IJraw	0.996	0.86 (0.84, 0.88)	-11.10 (-21.60, -0.86)	Yes	Yes	6.2C
	IJcorrected	0.996	0.86 (0.84, 0.89)	-10.30 (-20.81, -0.04)	Yes	Yes	6.2D
	IJKimura	0.996	1.07 (1.05, 1.10)	-11.86 (-24.98, 0.94)	No	Yes	6.2E
L|WinT	LI	0.988	1.05 (1.00, 1.10)	-41.14 (-65.57, -17.78)	Yes	Yes	6.2F
	IJraw	0.995	0.83 (0.81, 0.86)	-16.21 (-28.96, -3.83)	Yes	Yes	6.2G
	IJcorrected	0.995	0.84 (0.81, 0.86)	-15.41 (-28.18, -3.01)	Yes	Yes	6.2H
	IJKimura	0.995	1.04 (1.01, 1.07)	-18.20 (-33.81, -3.03)	Yes	Yes	6.2I
LI	IJraw	0.985	0.79 (0.76, 0.84)	16.49 (-3.36, 35.38)	No	Yes	6.2J
	IJcorrected	0.985	0.80 (0.76, 0.84)	17.34 (-2.59, 36.31)	No	Yes	6.2K
	IJKimura	0.985	0.99 (0.94, 1.04)	22.42 (-2.17, 45.83)	No	No	6.2L

Agreement between eight tested methods and the reference method

Both a regression analysis (Fig. 6.3 and Table 6.2) and a Bland-Altman analysis (Fig. 6.4 and Table 6.3) showed that all the semi-automated and automated methods used to estimate the TRL had a proportional bias when they were compared to the reference method. Looking at Fig. 6.4, the existence of such bias can be noticed by the significant positive correlation between the length differences and the TRL in a picture (see also Table 6.3). G|WinR, G|WinT and IJKimura were characterized by the lowest proportional bias, while IJraw and IJcorrected had the highest proportional bias (Tables 6.2 and 6.3). The regression analysis also showed that L|WinR, G|WinR, L|WinT and G|WinT were characterized by a fixed bias (Table 6.2). Interestingly, no bias was detected when the manual LI method was compared with the reference. Overall, all the tested methods tended to underestimate the TRL in the captured images (Fig. 6.3 and 6.4).

The lack of agreement between the tested methods and the reference was also evaluated by calculating limits of agreement (i.e., the limits within which 95% of the population values should lie) (Fig. 6.4 and Table 6.3). Although no fixed or proportional bias was detected for the manual LI method, the limits of agreement for the population of differences were large (149 cm below or 91 cm above the value given by the reference method) (Fig. 6.4E). Because of the existence of a proportional bias for the semi-automated and automated methods, we determined hyperbolic 95% confidence intervals (prediction interval) around the regression lines (Supplementary table 6.1). Our results showed that G|WinR (Fig. 4B) and G|WinT (Fig. 6.4D) had the narrowest prediction intervals, while L|WinT and IJKimura had the largest ones (Fig. 6.4C and 6.4H). Whether biased (semi-automated and automated methods) or not (manual LI method), all tested methods showed a lack of agreement with the reference method used in this paper. This was demonstrated by (1) a significant deviation of the Model II regression lines from the line of equal outcomes for the semi-automated and automated methods (Fig. 6.3 and Table 6.2) and/or (2) large 95% confidence intervals in Bland-Altman plots (Fig. 6.4 and Table 6.3).

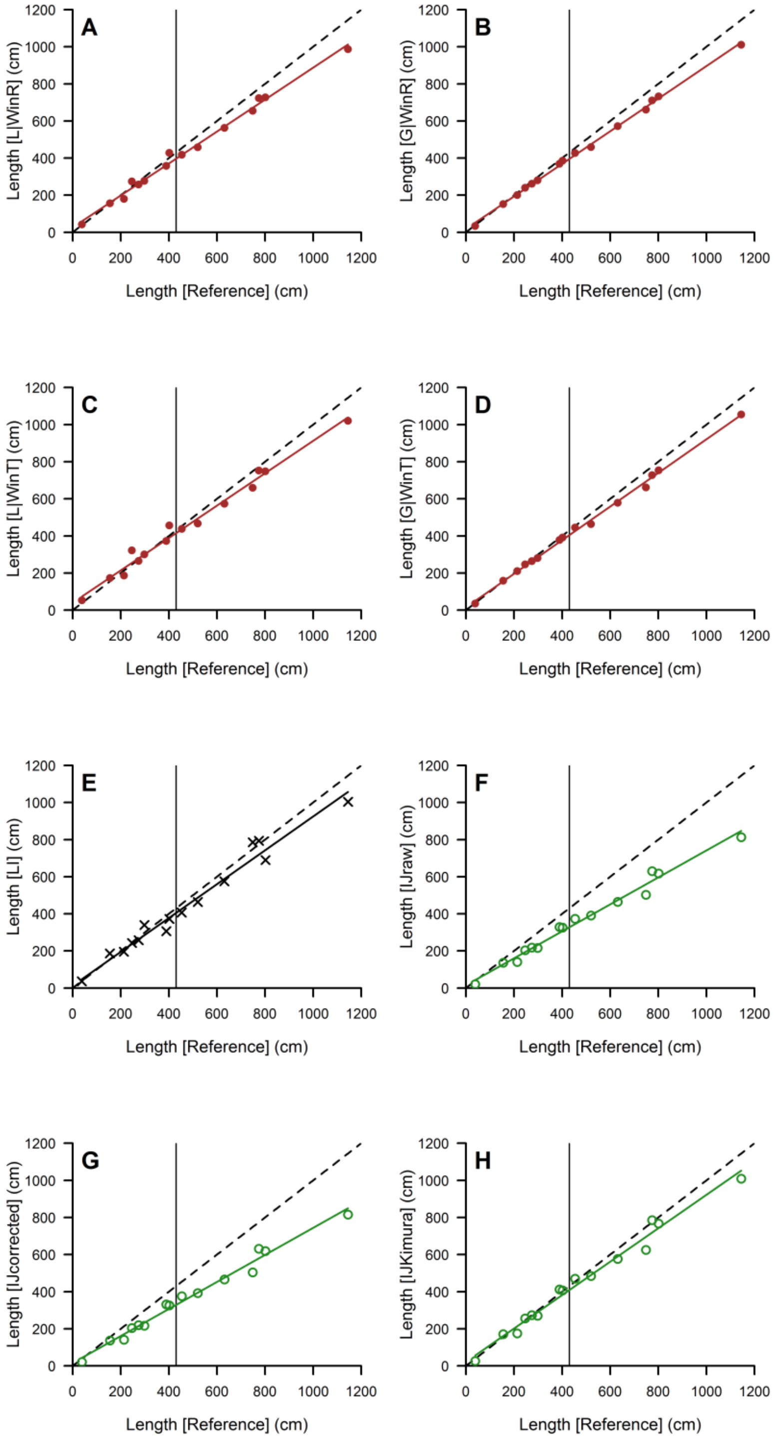


Figure 6.3. Evaluation of the accuracy of eight methods used for estimating the TRL of washed root samples. On each graph, both the ordinary least products regression line (solid line) and the line of equal outcomes (dashed line) were plotted. Filled brown dots, both fixed and proportional bias; open green dots, only proportional bias; black crosses, no bias. The vertical line corresponds to a RLD of 1 cm cm^{-2}. Abbreviations are explained in Fig. 1.

Table 6.2. Detection of fixed and proportional bias in eight methods used for estimating the TRL of washed root samples. The 95% confidence intervals (CIs) of the Model II regression coefficients are shown in parentheses (lower limit, upper limit) and were used to detect bias. If the 95% CI for the intercept did not include zero, there was fixed bias. If the 95% CI for the slope did not include 1.0, there was proportional bias. All correlation coefficients were significantly different from zero (*P*<0.001). Abbreviations are explained in Fig. 6.1.

Methods		**Correlation (Pearson)**	**Linear regression**		**Bias**		**Figure**
x	***y***		**Slope (95% CI)**	**Intercept (95% CI)**	**Fixed**	**Proportional**	
Reference	L|WinR	0.996	0.86 (0.81, 0.91)	26.33 (3.23, 48.19)	Yes	Yes	6.3A
Reference	G|WinR	0.999	0.88 (0.86, 0.90)	17.77 (7.16, 28.11)	Yes	Yes	6.3B
Reference	L|WinT	0.992	0.87 (0.81, 0.94)	39.93 (7.82, 69.72)	Yes	Yes	6.3C
Reference	G|WinT	0.999	0.91 (0.88, 0.93)	14.74 (1.34, 27.73)	Yes	Yes	6.3D
Reference	LI	0.986	0.91 (0.83, 1.01)	12.51 (-32.46, 53.24)	No	No	6.3E
Reference	IJraw	0.991	0.73 (0.67, 0.79)	15.02 (-13.18, 41.08)	No	Yes	6.3F
Reference	IJcorrected	0.991	0.73 (0.67, 0.79)	15.50 (-12.80, 41.65)	No	Yes	6.3G
Reference	IJKimura	0.991	0.90 (0.83, 0.98)	21.41 (-14.85, 54.83)	No	Yes	6.3H

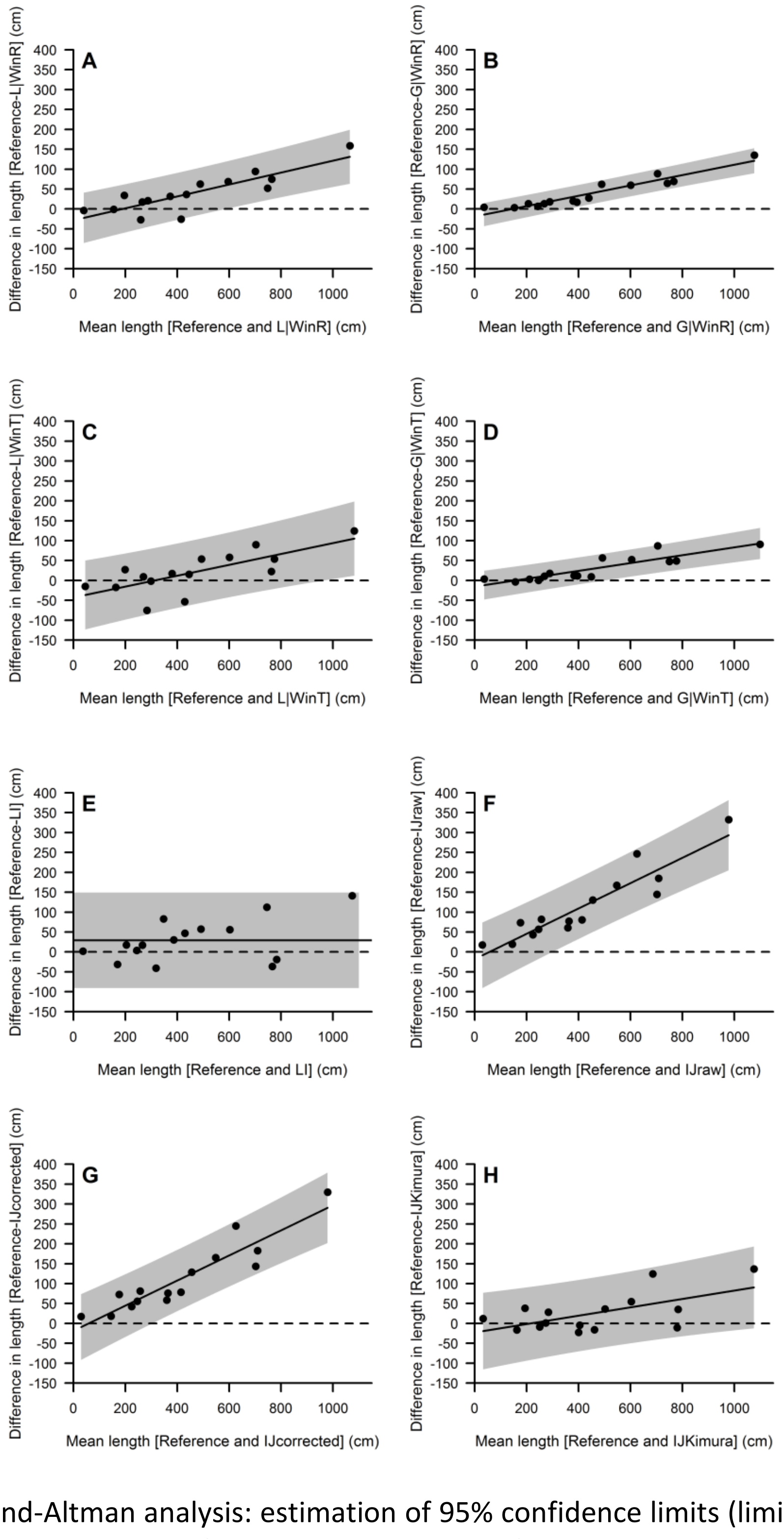


Figure 6.4. Bland-Altman analysis: estimation of 95% confidence limits (limits of agreement) for eight methods developed for estimating the TRL of washed root samples. In each graph showing a proportional bias, the ordinary least squares regression line was plotted (solid line) and hyperbolic 95% confidence limits (prediction interval) were constructed around the fitted linear model (grey area) using Eqn 3. When no proportional bias was detected, the limits of agreement were calculated using Eqn 2 (grey area) and a horizontal solid line shows the mean difference. Both overestimation (negative differences) and underestimation (positive differences) can be observed in the figures. Abbreviations are explained in Fig. 1.

Table 6.3. Bland-Altman analysis: estimation of 95% confidence limits (limits of agreement) for eight methods developed to estimate the TRL of washed root samples. The 95% confidence intervals (CIs) of the Model I regression coefficients are shown in parentheses (lower limit, upper limit). If the 95% CI for the slope did not include zero, there was proportional bias. In that case, we constructed hyperbolic 95% confidence limits (prediction interval) around the fitted linear model using Eqn 3. When no proportional bias was detected, the limits of agreement were calculated using Eqn 2. Abbreviations are explained in Fig. 6.1. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; ns, *P*>0.05.

Bland-Altman plot		**Correlation (Pearson)**	**Linear regression**		**Proportional bias**	**95% confidence limits**	**Figure**
x	***y***		**Slope (95% CI)**	**Intercept (95% CI)**			
(Reference+L|WinR)/2	Reference-L|WinR	0.852^{***}	0.15 (0.09, 0.20)	-28.44 (-57.40, 0.53)	Yes	Hyperbolic	6.4A
(Reference+G|WinR)/2	Reference-G|WinR	0.952^{***}	0.13 (0.10, 0.16)	-18.95 (-32.20, -5.69)	Yes	Hyperbolic	6.4B
(Reference+L|WinT)/2	Reference-L|WinT	0.738^{**}	0.14 (0.06, 0.21)	-42.90 (-83.03, -2.77)	Yes	Hyperbolic	6.4C
(Reference+G|WinT)/2	Reference-G|WinT	0.887^{***}	0.10 (0.07, 0.13)	-15.49 (-31.91, 0.93)	Yes	Hyperbolic	6.4D
(Reference+LI)/2	Reference-LI	0.486^{ns}	0.09 (-0.01, 0.19)	-13.38 (-66.72, 39.96)	No	(-90.98, 149.03)	6.4E
(Reference+IJraw)/2	Reference-IJraw	0.927^{***}	0.32 (0.24, 0.40)	-17.96 (-55.40, 19.47)	Yes	Hyperbolic	6.4F
(Reference+IJcorrected)/2	Reference-IJcorrected	0.925^{***}	0.32 (0.24, 0.39)	-18.49 (-56.03, 19.05)	Yes	Hyperbolic	6.4G
(Reference+IJKimura)/2	Reference-IJKimura	0.609^{*}	0.11 (0.02, 0.19)	-22.76 (-66.62, 21.10)	Yes	Hyperbolic	6.4H

When root lengths were computed with WinRHIZO^{TM}, we found a statistically significant positive correlation between the percent deviation of the estimates from reference values and the RLD (Fig. 6.5A-D). Our results were also strongly influenced by the thresholding method used for root skeletonization. The negative intercept values in Fig. 6.5A and 6.5C showed that small root samples were overestimated by WinRHIZO^{TM} when roots were skeletonized with the Lagarde thresholding method. This was particularly true when root lengths were calculated with L|WinT (Fig. 6.5C). This length overestimation of small root samples disappeared when a global thresholding associated with a local reclassification of grey level pixels was used (Fig. 6.5B and 6.5D). With the latter methodology, both the slope and the residual variance of regression models were lower than with the Lagarde method. In addition, the differences between estimates given by WinR and WinT were lower using this thresholding method (Fig. 6.5A-D). For a RLD of 1 cm cm^{-2}, G|WinR and G|WinT underestimated the TRL by 7.0 ± 6.2% and 4.7 ± 7.9%, respectively (predictions from linear models). When the TRL was estimated with the manual LI method (Fig. 6.5E) or with IJ_Rhizo (Fig. 6.5F-H), however, we did not find any significant correlation between the percent deviation of the length estimates and the RLD in the pictures. On average, the manual LI method underestimated the TRL by 4.2 ± 6.0% (Fig. 6.5E). Both IJraw (24.3 ± 4.8%) and IJcorrected (24.0 ± 4.8%) strongly underestimated the TRL of the analysed samples (Fig.

6.5F-G). With regard to IJKimura, it underestimated the TRL in the samples by 5.4 ± 6.1% on average when assessing accuracy related to the reference method (Fig. 6.5H).

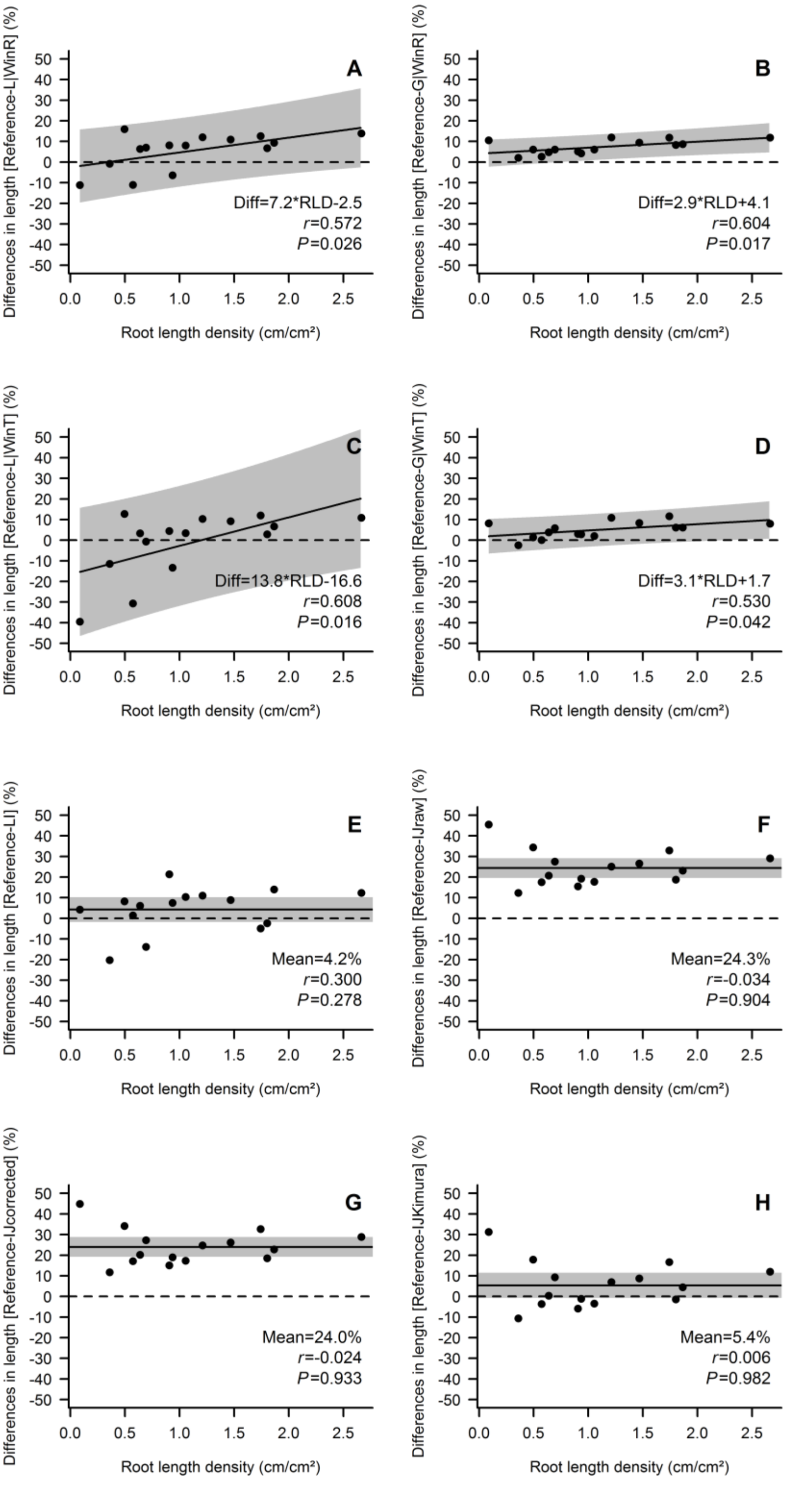


Figure 6.5. Effect of RLD in the images on the percentage deviation of the estimates from the values computed by the reference method. The Pearson`s correlation coefficient (*r*) and its associated P-value (*P*) are shown in each plot. When *r* was not significantly different from zero, the mean percent difference (solid line) and its 95% confidence interval (grey area)

were plotted. Otherwise, the Model I regression line (solid line) and the prediction interval (grey area) are shown. Both overestimation (negative differences) and underestimation (positive differences) can be observed in the figures. Abbreviations are explained in Fig. 1.

DISCUSSION

In the first part of this paper, we compared one manual (LI) and five automated methods (L|WinR, L|WinT, IJraw, IJcorrected, IJKimura) commonly used to estimate the total length of roots extracted from soil cores. Overall, we found a strong linear relationship between the lengths estimated by six tested methods. Previous reports showed that the lengths estimated with WinR and WinT are highly positively correlated (Wang & Zhang 2009; Pierret *et al.* 2013). Similar results were obtained when the root lengths computed by IJ_Rhizo were compared to those obtained with WinRHIZO^{TM} (Pierret *et al.* 2013). Nevertheless, out of 12 pairwise method comparisons performed in this study, 11 showed a significant deviation of the regression line from the line of equal outcomes (y=x). This result suggests that different methods of measurement can lead to different estimates of the same quantity. Supporting the results of Wang & Zhang (2009), we found that L|WinT tended to give higher length estimates than L|WinR. Interestingly, IJraw and IJcorrected gave lower length values than those computed with WinRHIZO^{TM} (L|WinR and L|WinT) or the manual LI method. Supporting the findings of Pierret and co-workers (2013), we found that IJKimura tended to give greater length estimates than those computed with WinRHIZO^{TM}.

In the second part of this paper, we were interested to test the accuracy of all methods listed in Fig. 6.1. Our results showed that all the tested methods tended to underestimate the TRL. In addition, all the semi-automated and automated methods were characterized by a proportional bias when they were compared to the reference method. The existence of a proportional bias means that the absolute difference between the values obtained with the tested methods and the reference increased with the amount of root material included in a sample. Previous reports showed that both the manual LI method (Tennant 1975; Goubran & Richards 1979) and image analysis algorithms (Zoon & Van Tienderen 1990; Smit *et al.* 1994) can underestimate the actual root length in a sample. With WinRHIZO^{TM}, even the use of the Lagarde's thresholding method developed for pale roots can lead to a strong underestimation of the TRL for unstained root samples (Bouma *et al.* 2000). Several factors could explain the overall underestimation of the TRL.

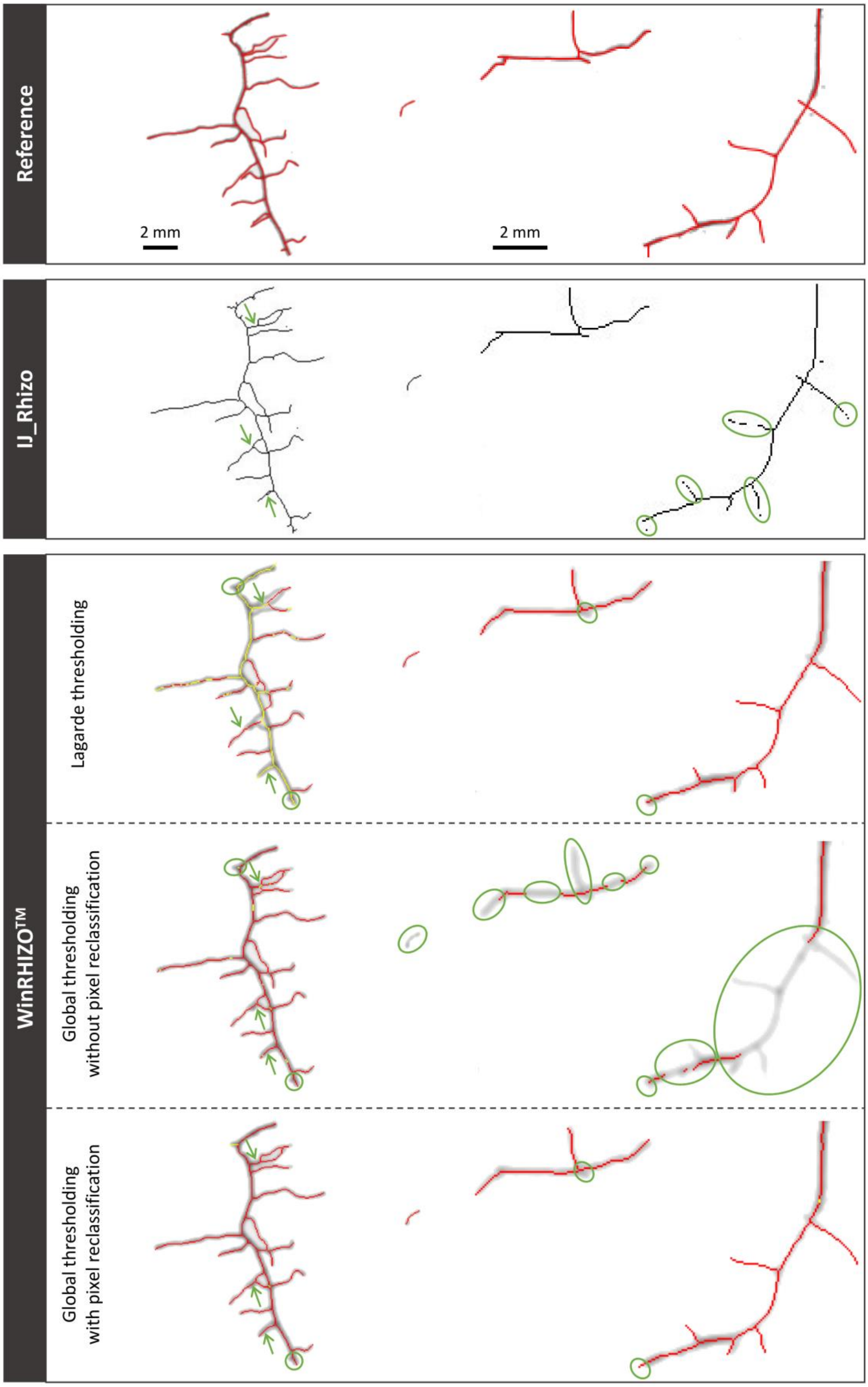

Reference
2 mm
2 mm
IJ_Rhizo
WinRHIZO™
Lagarde thresholding
Global thresholding without pixel reclassification
Global thresholding with pixel reclassification

Figure 6.6. Common root detection problems observed with the (semi-)automated methods discussed in this paper. Reference, roots were manually drawn with ImageJ. Both WinRHIZO^{TM} and IJ_Rhizo methods can misclassify pixels. Two causes are illustrated in this figure: (1) adjoining fine roots were detected as one single root of greater diameter (green arrows), and (2) automated thresholding methods failed to detect fine roots (green circles).

First, the manual LI method and some image analysis algorithms assume a random arrangement of roots on the scanned area resulting in a uniform distribution of orientations (Newman 1966; Ewing & Kaspar 1995). Therefore, a preferential orientation of roots can lead to inaccurate estimations of the TRL, particularly with the manual LI method that tends to overestimate root lengths when the orientation of roots is not random (Ewing & Kaspar 1995). Among the methods tested in this paper, both the Regent's method (Appendix C of the Regent's manual provided with the software; Himmelbauer *et al.* 2004) and IJKimura (Kimura *et al.* 1999) have a low sensitivity to preferential root orientation. Because (1) care was taken to randomly spread the roots on the scanning area, and (2) we mainly observed underestimation of the TRL, the influence of root distribution and orientation on the results did not seem to play a significant role in our study.

Second, the amount of root material included in a sample is also an important factor because a high RLD increases the occurrence of root overlaps (Fig. 6.6), leading to an underestimation of the TRL (Tennant 1975; Zoon & Van Tienderen 1990; Kimura *et al.* 1999; Costa *et al.* 2000). Using WinRHIZO^{TM}, both Bouma *et al.* (2000) and Himmelbauer *et al.* (2004) showed that the percent difference between length estimates and reference values increased with increasing RLD. The same pattern was observed by Goubran & Richards (1979) with the manual LI method, and by Zoon & Van Tienderen (1990) using image analysis. Our results confirmed these observations for the WinRHIZO^{TM} methods but not for the manual LI method. In order to avoid a too large underestimation of the TRL due to overlapping roots, some authors suggested to work with a RLD lower than 3 cm cm^{-2} (Bouma *et al.* 2000; Himmelbauer *et al.* 2004). In addition, if there is no interest in the study of root branching patterns, cutting the roots into small segments can decrease the number of crossings and adjoining objects when roots are spread all over the surface of the scanning area (Bouma *et al.* 2000). In our experiment, the roots were cut into smaller pieces and we worked with a RLD between 0.1 and 2.9 cm cm^{-2}. Because both WinR and the IJKimura are able to detect crossings and overlapping pixels, these methods have been advised by other people when the TRL has to be estimated for large root samples showing a high level of root overlaps (Appendix C of the Regent's manual provided with the software; Kimura *et al.* 1999; Himmelbauer *et al.* 2004).

Third, considering that a significant part of plant root systems are made of absorptive fine roots (Pierret, Moran & Doussan 2005; McCormack *et al.* 2015), the underestimation of TRL by image analysis algorithms could also be explained by the fact that low diameter roots were not detected because of the poor contrast between fine roots and background (Fig. 6.6). Using the reference method or the manual LI method, however, the roots were visually detected on a screen and even fine roots were easily separated from the background (Fig. 6.6). Therefore, if the observed underestimation was caused by the removal of fine roots on thresholded images, one can expect greater length estimates using the reference method or the manual LI method. In order to increase the contrast between roots and background, some authors recommended the use of a dye to stain the roots (e.g. 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; syn: neutral red; CAS 553-24-2), particularly when the samples contain a large proportion of fine roots (Wilhelm, Norman & Newell 1983; Bouma *et al.* 2000). In our study, we did not stain the roots because such an operation needs to be avoided when one wants to do chemical analyses of roots after the scanning. A compromise would be to stain only a subsample that will be used for root length measurement, and use the unstained part of the sample for chemical analyses. Collecting a representative subsample is not straightforward but protocols developed to estimate the TRL of large root samples can be found in the scientific literature (Goubran & Richards 1979; Schroth & Kolbe 1994; Costa *et al.* 2000).

Interestingly, the results computed with WinRHIZO^{TM} were strongly influenced by the thresholding method used to skeletonize the roots. When the RLD increased, the observed loss of accuracy was lower when the roots were detected with a global thresholding followed by a manual reclassification of grey level pixels in order to avoid missing roots. In addition, estimates given by WinR and WinT were much closer using this thresholding method. Although the interactive modification of grey level pixel classification increases the time required to analyse single images, root length measurements were more accurate and less variable using this approach. With regard to IJ_Rhizo, it has to be noted that the macro can be easily modified to test other thresholding methods available in ImageJ (Pierret *et al.* 2013).

Conclusion

In this study, we showed that the use of different methods to analyse the same images can lead to different results despite high correlation between the different methods. Therefore, care must be taken when measurements acquired on unstained root samples with different techniques are being compared. In addition, our results showed that all methods did not have the same accuracy and that using semi-automated or automated methods to estimate the TRL of unstained root samples can lead to underestimation.

Choosing a method for estimating the TRL of scanned root samples is not an easy task. Often, the selected method will be the result of a compromise between the desired level of accuracy and the time that one is able to invest in image analysis. Here, we advocate that the results obtained using image analysis will be influenced by the settings used for image acquisition and the method used to compute root length. On average, the manual LI method gave satisfactory results but its large limits of agreement suggest that it can lead to inaccurate estimations for single images. In addition, the manual LI method was more time consuming than the other methods used in this study. One has also to keep in mind that the manual LI method has sources of errors that image analysis software packages do not have such as involuntary omission of intersections, error in intercept interpretation using Tennant's guidelines, operator fatigue, and between-operator variation. When possible, we recommend to increase the contrast between fine roots and background during scanning using a dye to stain the roots. In addition, we recommend to avoid overlapping between roots by not exceeding a RLD of 1 cm cm^{-2}. Under these conditions, the best results were obtained with WinRHIZO^{TM} (global thresholding and interactive modification of pixel classification). Interestingly, we found that a good alternative to the commercial WinRHIZO^{TM} software package is the IJKimura method provided with IJ_Rhizo. In comparison with WinRHIZO^{TM}, IJ_Rhizo offers two additional advantages: it is free and open-source.

CHAPTER 7

Synthesis and Conclusions

The role of diversity and priority effects in grassland community assembly (chapters 2, 3, 4)

The *Priority effect experiment*, where we tested the effects of diversity and order of arrival on plant above and belowground performance (**chapter 2 and 3**), showed overall that priority effects can play a role during assembly. Having different groups of plants arriving before others affected aboveground biomass and species composition, while strong effects were found belowground. As shown in **chapter 2**, the aboveground biomass did not show the same pattern every year, but we found a trend of higher biomass production when legumes were sown first. These not persistent priority effects aboveground found in our experiment agree with Werner *et al.* (2016) in a Californian grassland restoration experiment. They found positive priority effects in the first years of the experiment, however long-lasting effects were verified only in part of the studied species. On the other hand, belowground data in the *Priority effect experiment* **(chapter 3)** showed a clear pattern in both studied years, with lower standing root length density in the plots where legumes were sown first when compared with plots where grasses were sown first. In 2014, this difference was even more evident, since the plots where legumes were sown first were also significantly different from the control treatments. The same pattern was found for root dry weight in 2014. Thus, the effects of sowing legumes first were more consistent below than aboveground. Considering that in the first year the L-first plots were composed mainly by legumes, one could argue that lower root length densities were found in these plots because legumes invest less in roots and more in shoots (Körner *et al.* 2008; von Gillhaussen *et al.* 2014). However, in the third year, we found an even stronger pattern of lower root length density and dry wright in the L-first plots, with a totally different species composition, which was mainly dominated by grasses. It suggests that somehow when legumes where sown first, plants (no matter from which group) would invest less in roots and more in shooting systems. These results showed that belowground effects may be key drivers during the creation of priority effects, since these priority effects belowground were consistent over time, and are in line with the few studies which tested the effects of order of arrival on roots (Körner *et al.* 2008; Moore & Franklin 2012; Orloff *et al.* 2013; Sikes *et al.* 2016). The same pattern was found by Körner *et al.* (2008) and Von Gillhaussen *et al.* (2014), who also manipulated the order of arrival of grasses, legumes and forbs, and found higher above and lower belowground biomass when legumes were sown first, compared with grasses sown first.

Both **chapters 2** and **3** showed that grasses and legumes performed differently, as drivers of grassland assembly, and that the extent to which they drove assembly did depend on their order of arrival. These results can be relevant to conservation of central European grasslands, which are endangered due to intensive agriculture and land abandonment. Sowing legumes first may be a good method for increasing aboveground biomass whilst maintaining diversity of central European grasslands. This can be an incentive to land managers to improve productivity but keep diversity at the same time. The dominance of grasses found in the end of the experiment would not be ideal for restoration aiming for diversity. However, the fact that all our treatments tended to be largely dominated by grasses shows that this was not related with the order of arrival treatments, but possibly with environmental factors, or with a lack of K in the soil. Recent research emphasized that studies in ecology should not only take into account long term experiments, but also the effects of the year of initiation, considering the weather conditions in the year when the experiment was implemented (Vaughn & Young 2010; Stuble, Fick & Young 2017). Possibly the environmental conditions in the year when the *Priority effect experiment* was established might have played a role in the community development. Thus, the potential for long lasting effects, as well as how the weather conditions in the beginning of the planting can affect the outcome of priority effects need further studies. Testing the year of initiation by starting the same field experiment every year could bring a better understanding of what is an effect of environment condition and what is related with priority effects.

If sowing legumes first results in less root productivity, one could inquire whether it would be an advantage if the goal would be to increase carbon storage in the system. De Deyn *et al.* (2011) showed in a long-term restoration planting that the amount of C and N was increased when a legume was added to the system. Thus, even though legumes do not invest much in roots (due to their great microbial activity), they allocate more carbon belowground to support the soil microbial community. However more studies are needed to understand the role of legumes in community composition, to evaluate carbon allocation for soil microbial community.

More studies are also needed to understand the mechanisms behind priority effects. We could already show how manipulating the order of arrival of different plant functional groups affects root productivity in the field. However, we could not separate species belowground as we could do aboveground. One of the limitations of studying roots is how difficult it is to separate the roots of different species. The green and red fluorescent protein method (Faget 2009; Faget *et al.* 2012, 2013b) used in the *Rhizotron experiment* and the DNA-based technique which allows investigation of species abundance in mixed root samples (Mommer *et al.* 2008, 2010, 2011) are opening opportunities to assign species to roots. These methods can be used to increase understanding of priority effects on root productivity and architecture.

We did not find evident effects of diversity in our experiment. Contrary to most of the BEF experiments, the high diversity plots resulted in higher number of species, but not higher aboveground biomass. As discussed in **chapter 2**, we believe that since we used the same dominant species in the low and the high diversity plots, we did not find evidence that diversity would promote a higher biomass. Moreover, we also did not find evidence that combining diversity and priority effects would result in higher productivity. We expected that the high diversity plots where legumes were sown first would be the most productive ones, however we did not find this, possibly because we did not find a diversity effect at all.

Soil properties (% C, % N, % K) did not change between the order of arrival treatments, showing that the species which arrived first did not change soil chemical properties. Effects of order of arrival were more evident in area B, which had a more fertile soil than area A. Priority effects are expected to be stronger in less harsh environments, since in this case species that first arrive grow more and better, giving less chances to the species arriving later (Chase 2003; Kardol *et al.* 2013; Vannette & Fukami 2014). Does it mean then, that in harsher environments priority effects would not play a role? And how harsh would the environment need to be so that it would unaffected by the plants arriving first? In recent studies in our working group we have found that dry acidic grassland in very sandy soils was also significantly affected by priority effects (data not shown here). However, more studies in different ecosystems and different environmental conditions should be conducted to answer these questions in a broad way. And once more studies are developed testing the strength of priority effects, we can apply this knowledge in restoration projects, such as by creating priority effects that promote facilitation, in order to drive assembly faster, and/or avoiding invasive exotic species (Lang *et al.* 2017).

In the paper reported in **chapter 4**, the methane yield obtained with the biomass collected in the *Priority effect experiment* showed that the order of arrival treatments affected indirectly the methane yield by affecting the relative dominance of plant functional groups. This study also concluded that since differences of methane yield were not large between the treatments, the composition of the community possibly played a bigger role in methane yield than which plant functional group was sown first. Thus, the dominance of the plant functional group that established in the plots actually drove the methane yield, where the highest values were obtained when legumes and grasses co-dominated.

According to these results, it is likely that grassland communities can be steered towards high methane yields by using priority effects. This seems to be a potentially valuable tool to maximize methane yields from grasslands. However, a restoration process aiming to return part of the ecosystem functions might be different from aiming at a high methane yield. Higher values of methane yield obtained by having grasses sown first would not be ideal when high biodiversity is desired. Therefore, how to properly combine a restoration strategy with bioenergy goals can be challenging and requires further studies.

Plant-plant interactions between legumes and non-legumes species (Chapter 5)

We found in the field a trend that plots where legumes arrived first were different from the others, showing that legumes species affected the system differently to non-legume forbs or grasses. In the *Rhizotron experiment* reported in **chapter 5**, where we interacted maize with a legume and non-legumes species, we found evidence that the identity and the spatial location of the neighbours played a role in plant-plant interactions, and that plant order of arrival modulated these interactions. Maize growing with a legume generally equated aboveground to the same outcome as not having a neighbour, while maize roots did not respond to the treatments when looking to the entire rhizotrons. However, when looking to the roots distribution between the right and left rhizotron zones of the rhizotrons, maize roots grew more towards the clover than towards the wheat neighbour. Root tended to forage toward the legume, which is the one that brings more nitrogen into the system. The higher leaf N found in the treatments containing a legume, when compared with the one without a legume neighbour, suggests N facilitation from legumes. In addition, the fact that when the three species were sown at the same time maize had lower leaf N shows that it did not have time to fix N as it had when it was sown before the other two species. These results are in line with other studies that found that having legume as a neighbour is an advantage due to direct and/or indirect facilitation (Temperton *et al.* 2007; Hauggaard-Nielsen *et al.* 2008; Duchene *et al.* 2017). Facilitative effects of having legume neighbours are well studied in agricultural science, but they normally focus on yield productivity (Li *et al.* 1999, 2001; Fan *et al.* 2006; Zhang *et al.* 2014). Results from the *Rhizotron experiment* helped to understand interactions between legumes and their neighbours, including the spatial location of these interactions and how roots distribute. Such findings have implications for intercropping in agricultural systems, BEF settings and restoration plantings. Knowing that interaction outcomes between plants depended on "who" is the neighbour and "when" they arrived in, can bring insights on species selection, on how to distribute them in experimental or agricultural sites, and on the usefulness or not of sowing different species before others. Results from the *Rhizotron experiment* complemented what was found in the *Priority effect experiment* bringing a better mechanistic understanding of how legumes interacted with non-legumes, since we could not test it in the field.

The importance of root data for this thesis (Chapter 6)

Roots collected in the *Priority effect experiment*, as well as the roots analysed in the *Rhizotron experiment,* contributed significantly to answering the main questions of this thesis. We brought another view to the study of priority effects showing belowground results, since the majority of the experiments that manipulated the order in which species arrive in the system reported only aboveground results.

Working with a large amount of roots during my PhD raised the question whether we can compare root length obtained from different techniques, resulting in a method paper which is reported in **chapter 6**. The total root length obtained in 2012 was estimated using the line intersect method (Newman 1966; Tennant 1975). To obtain the root length in 2014, we wanted to use an image analyses software packages, since it would be less time consuming than measuring by the manual method. However, we needed to test whether the manual and automated methods agree with each other, as well as their accuracy. For this reason, we compared measurements given by the line intersect and automated methods provided by WinRHIZO^{TM} and IJ_Rhizo **(chapter 6)**.

The results showed that: (1) the use of different methods to analyse the same images can lead to different results, (2) the studied methods did not have the same accuracy, and (3) the automated methods can underestimate the root length. After comparing different methods, we found that good estimates can be obtained with WinRHIZO^{TM}, but one of the methods in IJ-Rhizo (free and open source) can be an alternative to WinRHIZO^{TM}. Both methods allow faster analysis than the manual line intersect method. On average, the manual line intersect method gave satisfactory results, but can lead to bad estimations for single images. Overall, results showed that the compared methods can lead to different root length values. Based in these results, to be consistent, we used the same method to estimate root length in 2014 as we used in 2012, namely the manual line intersect method.

Priority effects and implications for restoration

Since communities can develop to several alternative states after disturbance, the direction to be followed would depend on the historical events, such as the order plants arrived in the site (Fukami 2015; Temperton *et al.* 2016). Understanding when priority effects occur, and how strong and persistent they are over time, should improve our knowledge of community assembly and therefore be highly relevant to ecological restoration, since we can push a community trajectory to a desired direction (Young, Petersen & Clary 2005; Temperton *et al.* 2016). Giving a head start to a certain plant species or a certain group of plants that would promote higher abundance, biomass and/or diversity is a way to promote positive priority effects. Comprehension of how priority effects happen in a certain ecosystem can bring good information on how this could be manipulated in order to increase the success of species introduced to restoration and on how better to restore the functions of the system, depending on what the restoration planting aims for. Predicting the outcome of priority effects in restoration projects is not an easy task considering that the outcome of a restoration project varies from site to site, year to year, species to species (Stuble *et al.* 2017). However, knowing the site and respecting the particularity of each ecosystem (as should be normal procedure in planning restoration), sowing a certain species or group of plants before the others can be a way to steer a community to a desired state.

Manipulating the order of arrival can be used as a restoration tool for example by increasing the biomass productivity (to motivate farmers to maintain grasslands), as well as to increase the success of desired species or native richness, and to avoid invasive species. Manipulating the order of arrival of native species, as was done in the *Priority effect experiment*, can have important consequences for restoration (Körner *et al.* 2008; van de Voorde *et al.* 2011; von Gillhaussen *et al.* 2014; Sarneel *et al.* 2016). Likewise, in places where invasive species threaten restoration managements, priority effects can also be used to drive the trajectory of a community by preventing exotic invasive species. Most of the experiments testing priority effects were interested in testing interactions between exotic and native species, and showed that the order of arrival plays a big role (Dickson *et al.* 2012; Cleland *et al.* 2015; Vaughn & Young 2015; Wilsey *et al.* 2015). Stuble & Souza (2016) found that native species paid a higher cost for arriving later. Thus, priority effects can be used to artificially drive assembly by avoiding negative effects and promoting positive ones, such as giving better chances to natives to establish before exotics. Wainwright & Cleland (2013) showed that exotic species have more plastic responses than native ones, so when arriving first, may supress the native species. Wilsey *et al.* (2015) found that exotic species reduced the biomass and diversity of late-arrivals much more than native species, showing strong priority effects by exotics. Grman & Suding (2010) also found strong priority effects of exotics on native species when working with Californian grasslands.

In restoration projects in Brazil, where restoration is required by law, native grasslands as well as tree seedlings growth are strongly hindered by African grassland species from the *Urochloa* genus (Sobanski & Marques 2014). Therefore, easy and inexpensive solutions are necessary to increase the likelihood that native species will grow better than the invasive grasses. To reduce this problem, a common practice is to use fast growing species, which can grow faster than the invasive grass, shading the area and avoiding the development of the invasive (Rodrigues, Brancalion & Isernhagen 2009). Practices like this are common in restoration projects in Brazil, however, they are not reported as priority effects, and possibly lack an experimental control. Studying successional dynamics in central Amazonian, Norden *et al.* (2011) found that differences in recruitment (caused by the species that first colonized) were the major drivers of alternative states. Thus, in environments which are surrounded by fragments containing native species, and which are therefore able to regenerate naturally, manipulating the first arrival species to avoid exotic species could allow subsequent natural regeneration.

Conclusions

The studies conducted in the *Priority effect experiment* closes some knowledge gaps. First, this was the first experiment to combine diversity and assembly in a natural environment reporting results of several growing seasons. Second, we targeted both above and belowground productivity collected in the field (despite the fact that these data are difficult to collect). Third, the analyses were focused on the amount of biomass stored above and belowground (carbon storage) and also on root architecture.

The importance of assembly rules was raised by Temperton *et al.* (2004), and followed by a ladder of experiments testing priority effects. The results presented in this thesis showed that: 1) different groups of plants arriving before others affected above and belowground biomass, 2) roots may be key drivers during the creation of priority effects, 3) interaction outcomes between plants depended on neighbour identity and location, modulated by the order they arrive in. Once the mechanisms of priority effects (changes in root-shoot ratio, asymmetric competition, changes in N cycling) are better understood, we will hopefully be able to implement it more effectively in restoration.

Even though the study of priority effects has been increasing in the last years, more investigations are needed in order to elucidate how priority effects may depend on geographical region or the ecosystem type studied, including how manipulating plant order of arrival can effectively improve restoration practices. In addition, more research is needed to understand how the strength of priority effects can change depending on the environmental conditions, and what the mechanisms are behind priority effects.

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CURRICULUM VITAE

EMANUELA WEHMUTH ALVES WEIDLICH

Nationality: Brazilian
Date of Birth: 10.12.1983
Address: Wallstr. 15, 21335, Lüneburg, Germany
Mobile: +49 1525 7550 115
e-mail: emanuela.weidlich@leuphana.de
www.leuphana.de/emanuela-weidlich.html
www.researchgate.net/profile/Emanuela_Weidlich
www.lattes.cnpq.br/8445154341580044

GRADUATION DETAILS

2017: PhD in Ecology – Leuphana University Lüneburg and Jülich Research Institute, Germany

2011: Master in Forestry Engineer - Federal University of Paraná and EMBRAPA Forest, Brazil.

2007: Graduation in Biology Sciences - Regional University of Blumenau, Brazil

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PROFESSIONAL EXPERIENCE

2013-2017: PhD Researcher – Leuphana University Lüneburg and Jülich Research Institute, Germany

2012-2013: Teacher at a Regional Faculty/College, teaching environmental courses, Fameblu, Brazil

2012-2013: Biology Teacher at Social Program from National Industry, teaching to Industry Employees (Concentrated High School), SESI/FIESC, Brazil

2010-2011: Master in Forestry Engineer - Federal University of Paraná and EMBRAPA Forest, Brazil.

2008-2009: Biology Teacher at Primary School, Shalom School, Brazil

2002-2007: Professional experiences at the University during Bachelor:

> Botanical Lab, working in a research group studying the effects of seeds size on the germination and development of native trees from Atlantic Forest
>
> Plant Biotechnology Lab, working in a research group studying resources allocation of native trees from Atlantic Forest
>
> Zoology/Taxidermy Lab, working as Curator and Taxidermist

Internships/Trainees

2015: University of Montana, USA (Restoration Ecology Lab)

2007: São Paulo University, Brazil (Plant Physiology Lab)

2006: EPAGRI- Agriculture Research Institute of Santa Catarina State, Brazil (Plant Biotechnology Lab)

2005: Natural History Museum of London, England (Botanical Department, Herbarium of *Flora Mesoamericana* Project)

COMPLEMENTARY COURSES

Alter Net Summer School in Ecosystem Services. Peyresq, France, 2014.

Environmental Inspection. Curitiba, Brazil, 2011.

Dendrology of rain forest. Manaus, Brazil, 2010.

Seeds ecology and dispersal. Blumenau, Brazil, 2004.

SCIENTIFIC COMMUNICATIONS

Peer-reviewed articles

EWA Weidlich, P von Gillhaussen, J Max, BM Delory, ND Jablonowsky, U Rascher, VM Temperton. Priority effects caused by plant order of arrival affect belowground productivity. Journal of Ecology (in press). doi: 10.1111/1365-2745.12829

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EWA Weidlich, P von Gillhaussen, VM Temperton (2015). Is a better understanding of assembly a way to help to reassemble communities for restoration? In: SER Conference, Manchester,UK.

Poster presentations at Conferences

EWA Weidlich, P von Gillhaussen, VM Temperton (2015). Above and belowground productivity of experimental grassland communities in the field during initial assembly. In: Rhizo 4 Conference, Maastricht, Netherland

EWA Weidlich, T Conradi, J Kollmann, VM Temperton (2014). Exploring plant community assembly for its potential for grassland restoration: the role of traits and functional diversity in assembling grasslands. In: Annual Meeting of the Ecological Society of Germany, Austria and Switzerland, Hildesheim, Germany.

EWA Weidlich, GR Curcio (2011). Desenvolvimento de espécies nativas sobre neossolo flúvico em área de restauração de ambiente fluvial. In: II Reunião Paranaense de Ciência do Solo

EWA Weidlich, GR Curcio, A Uhlmann E Zimmer, A Volkmann (2011). Sobrevivência de espécies arbóreas nativas em área de restaurãção de floresta aluvial no sul do Brasil. In: 61° Congresso Nacional de Botânica, Manaus.

Approval of own research

DECLARATION

I hereby certify that the submitted dissertation entitled “**Testing theories for ecological restoration: effects of plant-plant interactions and plant order of arrival on assembly and on above and belowground productivity**” has been written by me without using unauthorized aids. I did not use any aids and writings other than those indicated. All passages taken from other writings, either verbatim or in substance, have been marked by me accordingly.

I hereby confirm that in carrying out my dissertation project I have not employed the services of a professional broker of dissertation projects, nor will I do so in the future.

This dissertation, in its present or any other version, has not yet been submitted to any other university for review. I have not taken or registered to take another doctoral examination.

Lüneburg, July 2017 Emanuela Wehmuth Alves Weidlich