Climate change induced range-expanding plants

Aboveground and belowground interactions

Elly Morriën

Thesis committee

Thesis supervisor
Prof. dr. ir. Wim H. van der Putten
Professor of Functional Biodiversity
Laboratory of Nematology
Wageningen University

Other members
Prof. dr. Richard D. Bardgett   Lancaster University, UK
Prof. dr. Peter C. de Ruiter   Wageningen University
Prof. dr. Johannes A. van Veen   Netherlands Institute of Ecology/Leiden University
Prof. dr. Diana H. Wall   Colorado State University, USA

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ABSTRACT

Burning of fossil fuels has raised the level of atmospheric carbon dioxide, which contributes to global climate warming. As a result the mean earth surface temperature has increased faster in the past decades than in the previous thousands of years before. This rapid climate warming together with habitat fragmentation and other land use changes puts a major pressure on many plants and animals. They should either adapt to the warmer climate conditions or disperse in order to keep up with their optimal climatic conditions. Range expansion brings new interactions within the ecosystem in the new range. This can lead to potential benefits, for example range shifting species that do not encounter natural enemies in the new range might become invasive. Although invasive species are a well-studied phenomenon, there is relatively little known about the general mechanisms of biological invasions under climate change. In this thesis I focus on plant species that expand range due to current climate warming. I examined how these range-expanding plants interact with aboveground herbivorous insects and - mostly - how they establish belowground interactions with components of the soil food web. I examined how these interactions in the new range may play a role in the successful establishment of climate change induced range-expanding plants in plant communities of the new range. The focus of my study was on riverine (riparian) areas along the great rivers in the Netherlands, which are well connected with southern Europe by the Rhine and Rhine-Danube canal.

In the first experiment we examined exotic plant exposure to aboveground and belowground enemies. We used plants that originated from Eurasia (intra-continental range expanders) and plants that originated from other continents (inter-continental range expanders). We compared these exotic plants with phylo-genetically related natives. We grew the plants with and without non-coevolved polyphagous (generalist) herbivores, a locust *Schistocerca gregaria* and an aphid *Myzus persicae*. We also exposed all plants to a general soil community from the invaded range and compared their plant-soil feedback responses. Then I tested how individual plants responded to aboveground and belowground plant enemies and I compared this to their combined effects. I also tested whether the strength of aboveground control by generalist shoot-feeding insects was indicative of the strength of belowground control by plant-soil feedback.

In the next study I examined how the soil nematode community from the new range responds to exotic plant species compared to related native plants species. As a follow up, I determined the rhizosphere community composition of bacteria, fungi, arbuscular mycorrhizal fungi (AMF) and fusaria. All groups of microbes were analyzed qualitatively and the non-mycorrhizal fungal biomass and fusaria were also analyzed quantitatively. I tested the hypothesis that range-expanding plant species have a different rhizosphere microbial community composition than natives.

Finally, I compared the early establishment of range-expanding exotics and phylogenetically related plant species that are native in the invaded habitats. In a greenhouse I grew five range-expanding plant species and five related natives in sterilized and non-sterile inoculated soils from the new range, both alone and with a background community of plant species present in the invaded habitat. In the field, I grew the same plants species in artificially created sparse and dense plant communities. I tested whether range-expanding exotic plant species establish better under competition with native vegetation than phylogenetically related natives, because exotics may benefit from less negative interactions with the soil community compared to natives.

Conclusions:
- Range-expanding exotic plant species in riparian ecosystems are better defended against a non-coevolved generalist herbivore than congenerics that are native in the invaded habitats.
- Native plant species suffered more from belowground biotic interactions in their own soil compared to control soil than range-expanding exotic plants.
- Plant population control in both range-expanding exotics and congeneric native plant species may originate from additive effects of aboveground and belowground enemies.
- Temporal release from above- and belowground enemies can provide range-expanding exotics with a window of opportunity to become established and abundant in their new range.
- Exotic range-expanding plant species promote numbers of root-feeding nematodes less than congeneric natives, however, the overall taxonomic and functional nematode community composition is influenced by plant species rather than by plant origin.
- Range-expanding plants appear to have a rhizosphere soil community that differs from natives, but there was no causal link between rhizosphere microbial community composition and plant-soil feedback.
- Range-expanding exotic plant species did not show a benefit of their resistance or tolerance to soil-borne pathogens compared to congeneric natives when subjected to competition with a background plant community. Further studies need to examine if a difference may develop over time, when plant-soil feedback interactions build-up.
- In-depth studies on biotic interactions on a wide set of plant species in a wide range of habitats is needed to enhance knowledge about climate change effects on plant community composition and ecosystem functioning.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Since the industrialization of human society, burning of fossil fuels has raised the level of atmospheric carbon dioxide, which contributed to global climate warming (Vitousek *et al.*, 1997). As a result, over the past thirty years, the mean earth surface temperature has increased faster than in the previous thousands of years before (IPCC, 2007). This rapid climate warming together with habitat fragmentation puts a major pressure on many plants and animals (Thuiller *et al.*, 2005). They should either adapt to the warmer climate conditions or disperse in order to keep up with their optimal climatic conditions (Berg *et al.*, 2010). Dispersal might be constrained by habitat fragmentation, but also changing range itself brings a diversity of possible disadvantages. This involves new interactions within the ecosystem in the new range, new biotic and abiotic conditions in the new range, and finding an opportunity to become established into a new community. Changing ranges also may bring some advantages, for example when natural enemies are absent in the new range. Release of natural enemies gives the newcomers an opportunity to get established in an enemy-free environment, which might give them some competitive benefits in their new community. This may enable these species to become invasive in their new range (Mack *et al.*, 2000).

Although invasive species are a well-studied phenomenon, there is relatively little known about the general mechanisms of biological invasions under climate change (Levine *et al.*, 2003). In this thesis I focus on plant species that change their ranges due to current climate warming. I will examine how these range-expanding plants interact with aboveground herbivorous insects and I will give most attention to belowground interactions with components of the soil food web. I will examine how these changed interactions will play a role in the successful establishment of climate change induced range-expanding plants in plant communities that are native inhabitants of the new range.

Climate warming

The current mean average temperature of the earth's surface has increased by approximately 0.6°C compared to the 1951-1980 average (NASA). However, the poles are warming more rapidly than regions closer to the equator (Keeling & Garcia, 2002). The Arctic Ocean is expected to be ice-free by 2050, and parts of northern Canada and Alaska have experienced temperature rises of 10°C or more over the past 100 years (IPCC, 2007). Deserts are also expanding due to less rainfall in these areas. This is due to alterations in ocean streams and the El Niño cycles, which changes the distribution of precipitation (Holmgren *et al.*, 2007). Europe is also warming well above the global average. For example, in the Netherlands the average temperature has increased by almost 2°C over the past 50 years (KNMI, 2008). The consequences of global warming on global biodiversity are not entirely clear, however, there are many extinction incidences that can be linked to warming effects and lack of dispersal capacities or lack of suitable habitats (Thomas *et al.*, 2004a).
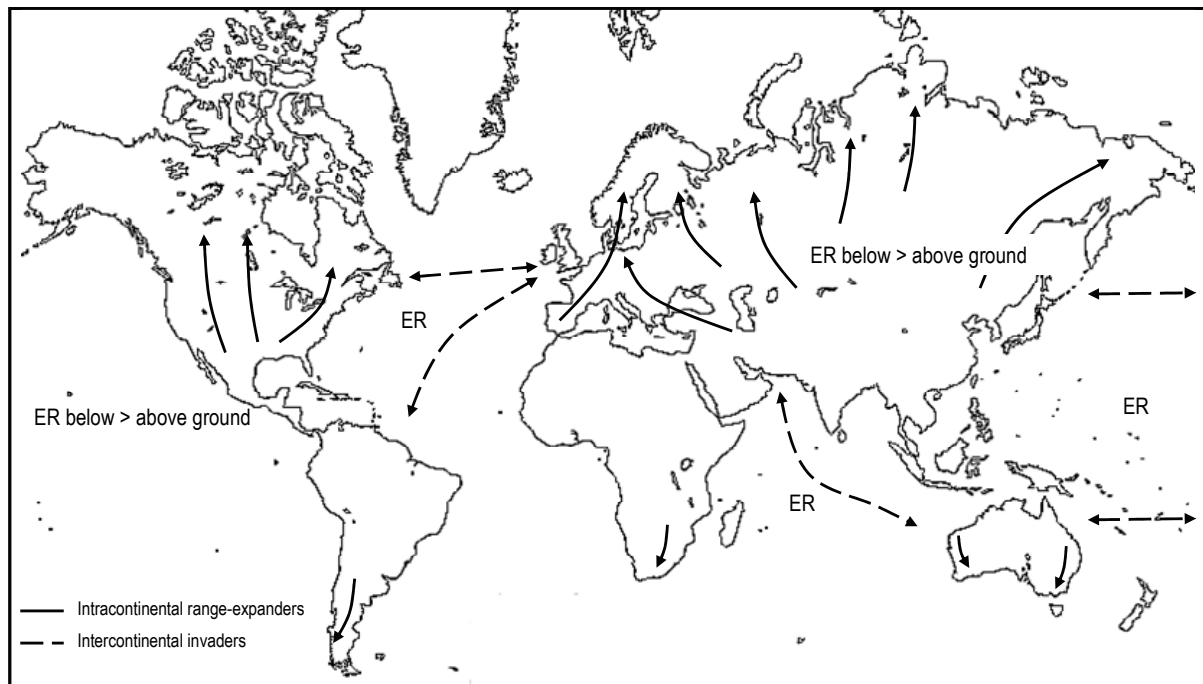
Figure 1 Differences between intercontinental invaders and intracontinental climate warming induced range expanders. The effects of enemy release in range expanders might be temporal: enemies may catch up, or existing generalist herbivores may switch hosts (which also occurs with intercontinental invaders on a longer time span). As aboveground enemies are more mobile than belowground enemies, range-expanding plants will be more likely to escape from their belowground than from their aboveground enemies. ER = enemy release. Figure is adapted from Morriën *et al.* (2010).

It is possible to make predictions on vulnerability of certain species for the effects of warming. Genetic adaptability, available niche space and dispersal capacities are in general good predictors of species survival under climatic change. Large-scale range expansions have already been recorded and a worldwide meta-study revealed a 6.1 km polewards shift per decade of a wide variety of plants and animals in the past decennia (Parmesan & Yohe, 2003). In the Netherlands, one quarter of the new arrived flowering plant species originate from more southern regions in Europe (Tamis *et al.*, 2005). Some of these species were accidentally introduced, others intentionally as ornamental plants. Nevertheless, many other species have made it to the Netherlands by natural dispersal from the south. The arrival of new exotic plant species is expected to generate novel interactions among exotic and native plant species (van der Putten *et al.*, 2004), as well as novel interactions with food web components of the invaded range.

Range expansions in plants

Intracontinental range expansions can occur within any continent (Figure 1). Most likely intracontinental range expansions can also occur on the southern hemisphere, but on a smaller scale due to less land mass available for vascular plants. In their new environments, intracontinental range-expanding plants are exposed to

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aboveground and belowground biotic interactions different from their original range. The same phenomenon of range expansion also occurs with plants that have been introduced first from other continents before moving pole wards (Figure 1). These species are called intercontinental range-expanding plants and a number of them are highly invasive in their new range. For this thesis the focus is on intracontinental range expansion, but since much of the ecology of intracontinental range expanders in their new habitats is unknown, we will make use of the basic concepts developed for intercontinental plant invasions.

The main gateways of intracontinental plants into new ranges are via ruderal areas such as river banks, road verges, and railway tracks. Therefore, the traits of the climate change induced intracontinental range expanders are those of quick colonizers, wind or water dispersers. Many of these plants first establish along the dispersal corridors, from which they may spread into adjacent habitats. Temperate regions are now colonized by both intercontinental and intracontinental range-expanding plant species (Figure 2). Intracontinental range expanders may, or may not have the same invasive traits as intercontinental invaders and they most likely are facing biotic interactions that differ from those in their native range. The exposure of the range expanders to biotic interactions in the new range can differ from related native species, for example because their associated aboveground and belowground species do not migrate at the same rate (Berg *et al.*, 2010).

The mechanisms that facilitate establishment of intercontinental invaders may vary from little competition on disturbed sites, to enhanced benefits from low-specific symbionts and reduced exposure to natural enemies (Colautti *et al.*, 2004). On the other hand, the abundance of intercontinental exotics may be reduced when not recognized by symbionts or by predators of their enemies (Verhoeven *et al.*, 2009). Once the intercontinental invaders have become established, they may change aboveground and belowground biotic interactions in their new range, because abundant dominance strongly influences the quantity and quality of food supply for invertebrates, vertebrates and soil microbes (Parker & Hay, 2005).

Intercontinental exotic plants can introduce novel compounds, to which native plants and other biota have little or no tolerance or defenses (e.g. Cappuccino & Arnason, 2006). Intercontinental invasive plants may also alter detritus chemistry, which influences nutrient cycling in the new range (Ehrenfeld, 2003). Therefore, besides changing biotic interactions in the new range, the intercontinental invaders may also be able to alter resource quality for root-feeding soil biota and decomposers. This then may feed back to the capacity of the intercontinental plant species to become abundant, although there are too many exceptions to consider this effect on nutrient cycling a general pattern (Meisner *et al.*, 2009). Finally, selection on plant traits may differ between the native and the new range of intercontinental exotics (Blossey & Nötzöld, 1995; Joshi & Vrieling, 2005). All these issues have been relatively well studied for invasive plant species from intercontinental origin, whereas little knowledge exists on intracontinental range expanders.

Figure 2 'New' plant species in the Netherlands. *Impatiens glandulifera* (above left) is a 'classic exotic invader' native to Himalayan mountains, introduced as ornamental plant and currently a pest species in many parts of Europe (photo: Jinze Noordijk). *Solidago gigantea* (above right) is an 'intercontinental range expander' native to North-America, introduced in Europe and expanding its range northwards within Europe (photo: Nico de With). *Artemisia biennis* (below left) is an 'intracontinental range expander' originating from Asia, introduced in Central- and South-Europe, and now occurring in the Netherlands (photo: Jinze Noordijk). *Tragopogon dubius* (below right) is an 'intracontinental range expander' native to South-Europe currently expanding its range into the Netherlands (photo: Co Morriën).

Plant-enemy interactions aboveground

Aboveground enemies are capable of controlling plant population growth by their consumption of shoot material (Crawley, 1997). The Enemy Release hypothesis (ER) explains successful establishment and performance of exotic plant species in their new ranges as a consequence of reduced enemy pressure (Figure 1) (Keane & Crawley, 2002). To what extent the exotic plant experiences enemy release depends on the kind of release, and the nature of novel biotic interactions in the new range.

Specialist enemies can exert strong top-down control since they have coevolved with their host plant. A loss of specialists in particular would release exotic plants from top-down control, thereby leading to invasion (Mitchell & Power, 2003; Wolfe, 2002). However, upon arrival in new ranges, exotic plants encounter novel enemies as well. Successful use of this new resource by native herbivores may constrain the invasion process, which is also known as biotic resistance (Parker & Hay, 2005). Generalist herbivores are present in all habitats and colonize new hosts faster than specialists (Strong *et al.*, 1984). Whether exotic plants experience reduced enemy impact, is a balance between the impacts from the enemies lost from their original range, and the enemies gained in their novel range.

Although many aboveground herbivorous insects have the ability to fly and actively explore distant host patches, they do not always follow their host into the more northern ranges. Climatic barriers may differ for plants and insects and changes in climatic conditions can also exert different effects on insect species (Bale *et al.*, 2002). Where some insects will benefit, others will be more constrained in their performance. Also, as range-expanding plants are encountering novel conditions and interactions, so will their enemies.

Plant-soil interactions

The ER hypothesis can also be applied to belowground plant-enemy interactions. For example, in the new range abundant exotic plants can be released from their original soil pathogens, while native plants accumulated local pathogens at a much higher rate (Klironomos, 2002). Following from this, Mangla *et al.* (2008) found that exotic plants attracted pathogens that only negatively affected native community members, but not the exotics themselves. Thus, aside from the benefit of having lost important soil pathogens in the invasion process, disrupting of the local soil community could contribute to exotic plant performance as well (Eppinga *et al.*, 2006). Release from belowground enemies seems more likely than release from aboveground enemies, since belowground enemies are less mobile than the ones aboveground. It has been shown that range-expanding exotics experience a net lower pathogen pressure in their new range, than in their native range (van Grunsven *et al.*, 2010). A reduced exposure to belowground pathogens can contribute to increased performance of range-expanding exotic plants in contrast to resident community members.

Aside from pathogens plants also have belowground mutualistic relationships with for example arbuscular mycorrizhal fungi (AMF). AMF are commonly regarded as generalists and are present in most habitats, so that alterations in these mutualisms are supposed to be less influential on plant invasions (Richardson *et al.*, 2000). Whether or not this is a general pattern during range shift remains to be investigated. In some cases, local mutualistic symbionts even can be strongly suppressed by invasive non-mycorrhizal exotic plants. These exotics may use an active process by which phytochemicals are excreted that suppress AMFs in

the soil (Stinson *et al.*, 2006) or a passive process by which the abundant non-mycorrhizal exotics do not support the AMF in the soil leading to their decline (Vogelsang & Bever, 2009). In both cases the capacity of native mycorrhizal-dependent plant species to persist and survive in the invaded community were reduced. This has been studied for intercontinental exotic plants, however, not for intracontinental range expanders.

Nematodes

Nematodes are important components of the soil food web, driving ecosystem processes (Karssen *et al.*, 2010; Yeates, 1999), influencing crop production (Yeates & Newton, 2009) and plant community structure (e.g. van der Putten & van der Stoel, 1998; Verschoor *et al.*, 2002; Yeates, 1999). Besides root-feeding nematodes, there are nematodes feeding on protozoa, fungi, bacteria, mites and other soil nematodes (Yeates *et al.*, 1993a) (Figure 3). As nematodes are predominantly soil inhabitants, they are supposed to have less dispersal capacity and to respond less to climate warming by range expansion than many plant species (Berg *et al.*, 2010). A reduced diversity (Brinkman *et al.*, 2005) or density (de la Peña *et al.*, 2008; de Rooij-van der Goes, 1995) of root-feeding nematodes can result in less feeding pressure on range-expanding plant species compared to natives. This might explain why exotic range-expanding plants receive less negative feedback effects from the soil community of invaded habitats than phylogenetically related native plant species (van Grunsven *et al.*, 2007).

Root-feeding nematodes differ in their pathogenicity and the amount of damage caused to plants. Pathogenicity relates to the capacity to cause disease or damage and virulence relates to the capacity to circumvent resistance of the host plant (Trudgill, 1991). The amount of damage depends on the nematode feeding strategy. Most ectoparasites (e.g. *Trichodorus*, *Tylenchorchynchus* spp.) are necrotrophic. The injected saliva liquefies the cytoplasm, which is rapidly ingested and usually kills the host cell. The nematode then moves to a new cell and repeats the process. Such behaviour limits the possibilities for effective induced resistance against those plant parasitic nematodes (Zuckerman & Rohde, 1981). Migratory endoparasites (e.g. *Pratylenchus* spp.) invade the plant and feed inter- or intracellularly. These species have a specific host range and high reproduction rates and are extremely damaging (Trudgill, 1991).

Sedentary endoparasites are obligate biotrophs. The invasive juveniles lose their mobility and require the cells on which they feed to remain alive. Therefore they change plant cells in ways that improve the supply of food. These responses range from the induction of a single giant cell to several giant cells (e.g. *Meloidogyne*), or a multinucleate syncytium (e.g. *Globodera* and *Heterodera*). As a consequence of this close association of sedentary endoparasitic nematodes with their hosts, the developing females have no need to retain their mobility and therefore can become much enlarged, with increased reproductive rates. Nematodes within this group are
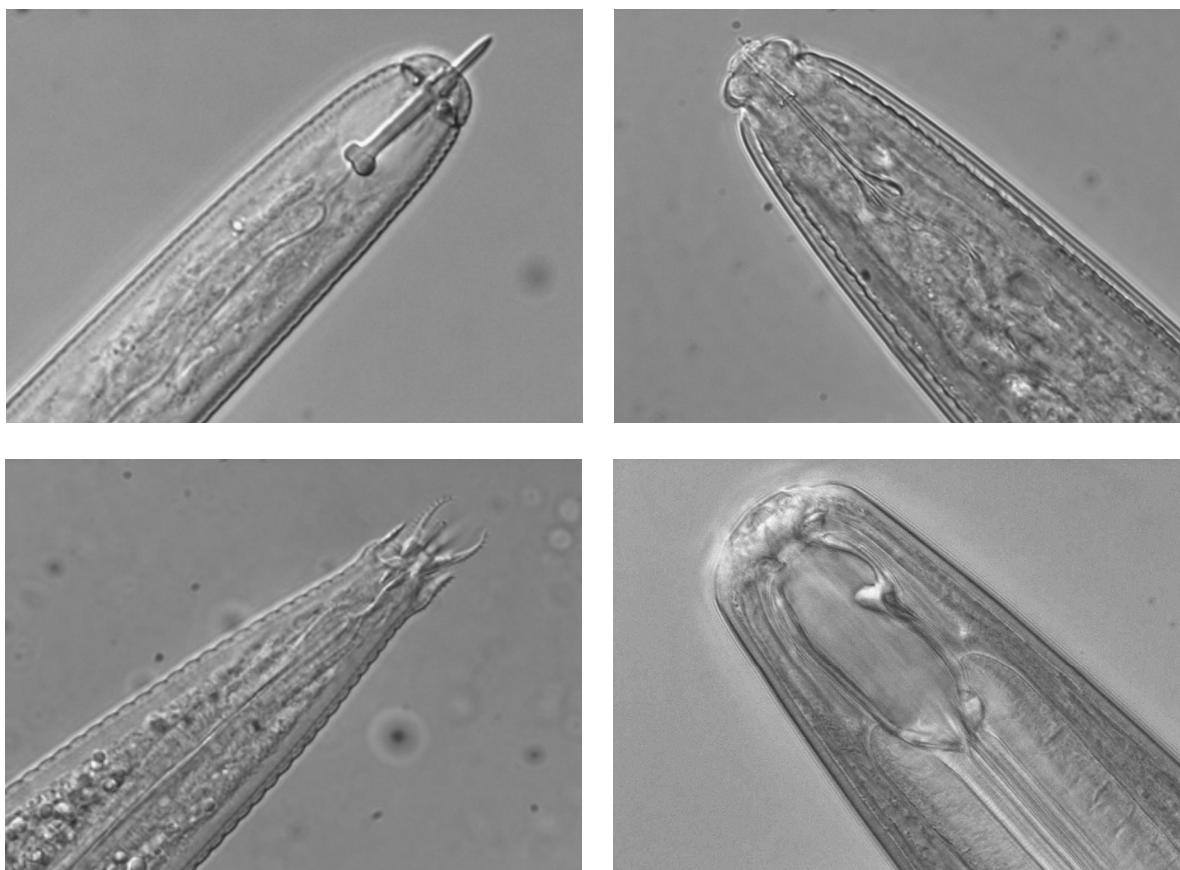
Figure 3 Mouth parts of different nematode feeding guilds. *Globodera rostochiensis* (Above left) is a sedentary endoparasitic root-feeding nematode that feeds on plant roots (Photo: Hein Overmars). *Tylencholaimus sp.* (Above right) is a fungivorous nematode species (Photo: Hanny van Megen). *Acrobeles* (Below left) is a bacterivorous nematode species (Photo: Hanny van Megen). *Monochus truncates* (Below right) is a carnivorous nematode species which feeds on other nematodes (Photo: Hanny van Megen).

among the most damaging to plants (Trudgill, 1991). Resistance to pathogens describes the effects of host genes that restrict or prevent nematode multiplication in a host species. Tolerance of damage is independent of resistance and relates to the ability of a host genotype to withstand or recover from the damaging effects of nematode (Trudgill, 1986).

Bacteria
Root exudates and rhizo-deposits form the substrates for rhizosphere bacteria, and it has been recognized that the composition of these substrates can differ between plant species (Nelson, 1990). These differences in root-derived substrates are believed to explain the plant specific rhizosphere bacterial communities that have been observed for different plant species under similar conditions (Carney & Matson, 2005; Hartmann *et al.*, 2009; Kowalchuk *et al.*, 2002; Marschner *et al.*, 2001). However, the root exudate composition is also affected by growth conditions in the soil, e.g. pH, nutrient limitation, soil moisture and exposure to pathogens (Yang & Crowley, 2000). Furthermore, different soil types are known to possess different bacterial communities, which appear to be independent of plant species composition

(de Ridder-Duine *et al.*, 2005; Kuske *et al.*, 2002; Singh *et al.*, 2007) so the plant community and the soil type both shape the structure and function in the rhizosphere of bacterial communities in particular and soil biota in general (Berg & Smalla, 2009; Eisenhauer *et al.*, 2011).

Fungi

Fungal communities also respond to plant community composition and processes in the rhizosphere (de Boer *et al.*, 2006; Gomes *et al.*, 2003). Since soil fungi contain groups with saprophytic, pathogenic but also plant symbiont functions, general and specific fungal groups are valuable to study in a plant-soil community feedback context. The feedback effects to plants of soil microbiota from a range invaded by an exotic plant is likely to be neutral or positive because of the potential for the invader to accumulate mutualistic fungi in the absence of host-specific soil pathogens (Bennett *et al.*, 2006; Callaway *et al.*, 2004; Pringle *et al.*, 2009). On the other hand, in some cases local mutualistic symbionts are suppressed by non-mycorrhizal exotic plants. Arbuscular mycorrhizal fungi are capable of suppressing negative effects from root pathogens on the host plant where they have colonized the roots (Borowicz, 2001; Newsham *et al.*, 1995). Mycorrhiza can also alter soil structure and therefore the interactions between plant roots and soil structure but also other parts of the soil microbial community, such as the bacterial community (Rillig & Mummey, 2006; Rillig *et al.*, 2006).

The genus of *Fusaria* spp. includes a number of economically important plant pathogenic species that cause a lot of damage on crop plants such as bananas and asparagus. They are a large genus of fungi widely distributed and relative abundant members of the soil microbial community in association with plants (Booth, 1971). In addition and in contrast to the host-specific tendency of pathogenic microbes, many mycorrhizal fungi tend to infect a broad range of plant hosts (Eom *et al.*, 2000).

Plant-soil feedback

A common way to study the net effects of soil communities on plants is by testing plant-soil feedback. According to this approach, plant growth in own soil is compared to a control soil; negative plant-soil feedback indicates net pathogenic activity, whereas positive plant-soil feedback indicates that the net growth enhancing effects of symbionts and decomposer organisms overrule the pathogenic effects (Figure 4) (Bever *et al.*, 1997).

Soil community feedback is a widespread phenomenon that can contribute to plant diversity, coexistence and also plant invasions (Bever, 2003; Bever *et al.*, 1997; Klironomos, 2002; Petermann *et al.*, 2008; Inderjit & van der Putten, 2010). When a plant species enters a community, soil feedback becomes increasingly negative when time since invasion proceeds (Diez *et al.*, 2010). Soil microbes have profound effects on plant growth and survival through pathogenic effects, root-fungus mutualisms and by driving the nutrient cycles on which plants depend (Mitchell & Power, 2003;
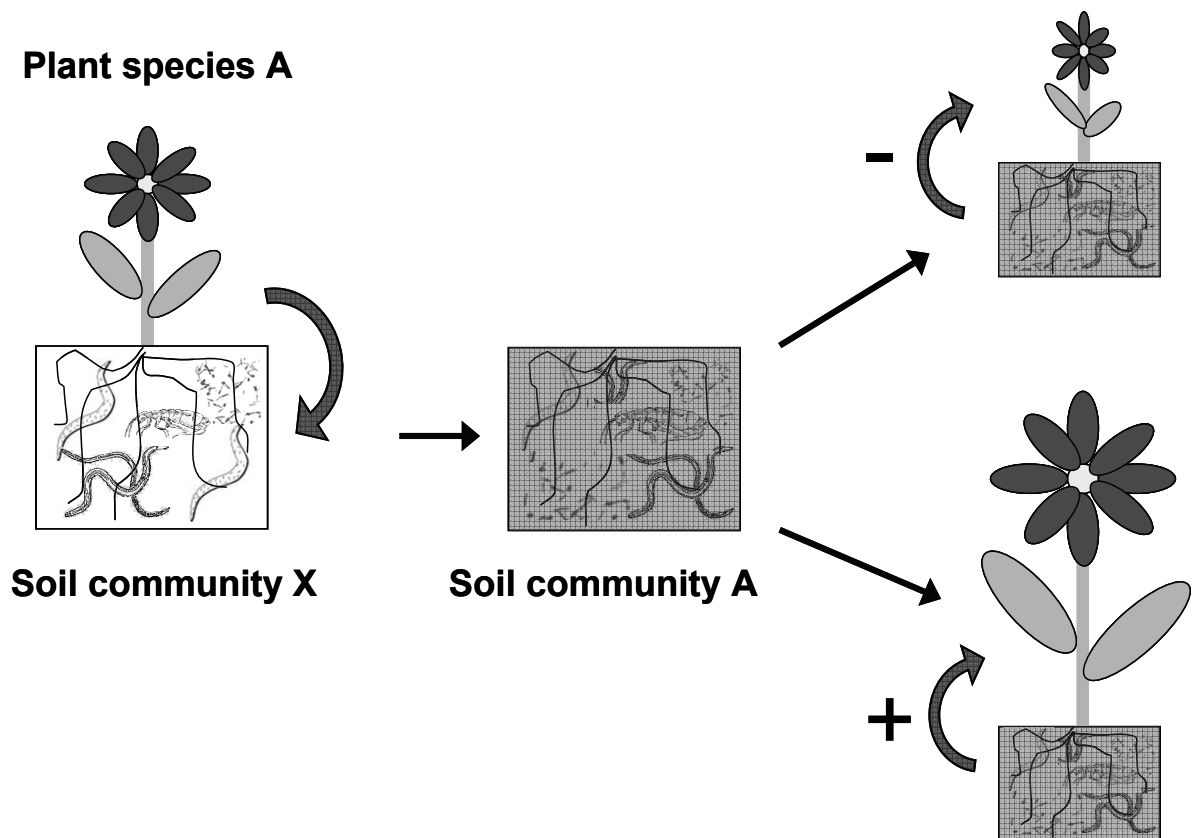
Figure 4 Schematic view of the plant-soil feedback approach. Plant species A influences soil community X so that it changes to a soil community specific for plant species A. When plant species A grows where soil community A has developed, this soil community can either have a net negative or a net positive effect on plant species A depending on the net effect of accumulation of pathogens or mutualists.

Newsham *et al.*, 1995; Packer & Clay, 2000, Sanon *et al.*, 2009). Exotic plant species can alter the microbial community in the rhizosphere (Callaway *et al.*, 2004; Kourtev *et al.*, 2002; Kourtev *et al.*, 2003) through root exudates of the plant (Broeckling *et al.*, 2008). However, it seems difficult to draw causal links between exotic plant invasion, root exudation, soil community changes and soil feedback build-up (Batten *et al.*, 2006). Soil-feedback effects can be effective both in the short term, as well as long term through changes in microbial communities (Kulmatiski & Beard, 2011).

Aim and thesis outline

The main aim of this thesis is to study the effects of aboveground and belowground interactions with climate induced range expanding plants. We examined how these interactions could contribute to the success of range expanders in their new range compared to related native plants that co-occur in similar habitats. Thereby, we searched for general mechanisms that lead to the successful establishment of these climate induced range expanders from warm into previous colder regions. This involves comparisons with invasion strategies, such as enemy release that plays a role in the invasion process of exotic plants that are introduced from other

continents; we have studied whether these strategies also apply to intracontinental range-expanding plant species.

In CHAPTER 2 We examine whether range-expanding plant species in a riparian habitat were better defended against shoot and root enemies than related native plant species growing in the same area. We test the hypothesis that range-expanding plant species suffered less from aboveground generalist shoot herbivores and from their soil community.

In CHAPTER 3 I test how the aboveground effect of generalist herbivores and the belowground effect of the soil community add up. I test the hypothesis that aboveground and belowground enemy effects add up in a linear fashion and that this type of addition does not differ between natives and exotic range expanders. I also test whether the strength of aboveground control by generalist herbivores is indicative of the strength of belowground control by plant-soil feedback.

In CHAPTER 4 I focus on the effect of soil nematodes on exotic range expanders and *vice versa*. I expect that range expanding plant species have fewer root-feeding nematodes than related native plant species, as predicted by the enemy release hypothesis. Besides, I expect that range expanders affect the taxonomic and functional composition of the nematode community, but that these plant origin effects would diminish with increasing trophic position of the nematodes in the soil food web (Scherber *et al.*, 2010).

In CHAPTER 5 I examine the soil microbial community composition in the rhizosphere of range-expanding exotics and related native plant species. I test the hypothesis that exotic range-expanding plants have a different rhizosphere community than native plants and that quantitatively, range expanders have fewer plant pathogenic microbes in their rhizosphere than their related native counterparts.

In CHAPTER 6 I compare early establishment of range expanding exotic plant species and phylogenetically related plants that are native in the invaded habitats. I tested this in a greenhouse and in a field experiment where native and range expanding plants are placed in a competition-free environment or in competition with a background community from a native riverine vegetation. In the greenhouse I also tested the effect of the soil community by comparing sterile with non-sterile soil in combination with competition. I test the hypothesis that range-expanding exotic plant species establish better under competition with native vegetation than phylogenetically related natives, because exotic plants benefit from less negative interactions with the soil community than natives.

Finally, in CHAPTER 7 I discuss and synthesize the main findings from this thesis. I discuss the aboveground herbivore effects and soil feedback effects in native and range-expanding plant species and the role of soil community composition in the success of range-expanding plant species. Then I conclude by discussing competition of range-expanding plant species with a natural background vegetation community in the field situation and I present some ideas for future research.

Chapter 2

SUCCESSFUL RANGE-EXPANDING PLANTS EXPERIENCE LESS ABOVEGROUND AND BELOWGROUND ENEMY IMPACT

Tim Engelkes, Elly Morriën, Koen J.F. Verhoeven, T. Martijn Bezemer, Arjen Biere, Jeffrey A. Harvey, Lauren M. McIntyre, Wil L.M. Tamis & Wim H. van der Putten

Abstract

Many species are currently moving to higher latitudes and altitudes (Walther *et al.*, 2002; Parmesan & Yohe, 2003; Pearson & Dawson, 2003). However, little is known about the factors that influence the future performance of range expanding species in their new habitats. Here, we show that range expanding plant species from a riverine area were better defended against shoot and root enemies than related native plant species growing in the same area. We grew fifteen plant species with and without non-coevolved polyphagous locusts and cosmopolitan polyphagous aphids. Opposite to our expectations, the locusts performed more poorly on the range expanding than on the congeneric native plant species, whereas the aphids showed no difference. The shoot herbivores reduced biomass of the native plants more than of the congeneric range expanders. Also the range-expanding plants developed fewer pathogenic effects (Klironomos, 2002; van Grunsven *et al.*, 2007) in their root zone soil than the related native species. Current predictions forecast biodiversity loss due to limitations in the ability of species to adjust to climate warming conditions in their range (Warren *et al.*, 2001; Thomas *et al.*, 2004a,b). Our results strongly suggest that the plants that shift ranges towards higher latitudes and altitudes may include potential invaders, as the successful range expanders may experience less control by aboveground or belowground enemies than the natives.

Introduction

Range expansion is a key adaptive feature of species in response to changes in climate, habitat availability and other limiting factors (Walther *et al.*, 2002; Parmesan & Yohe, 2003; Warren *et al.*, 2001; Thomas *et al.*, 2004a,b; Lovejoy & Hannah, 2005; Brinkhuis *et al.*, 2006). Currently, a number of species are showing rapid range expansion from warmer into previously colder biomes (Tamis *et al.*, 2005). As not all species have the same range shift capacity, ecological interactions may become disrupted as the community species pool changes (Lovejoy & Hannah, 2005). Rapid range expansion and the loss of control by natural enemies are key features of invasive species (Levine *et al.*, 2006; Keane & Crawley, 2002). However, very few studies have actually investigated range expansion in relation to enemy exposure (van Grunsven *et al.*, 2007; Menendez *et al.*, 2008). The aim of our study was to examine how rapidly range-expanding plant species are defended against aboveground and belowground natural enemies as compared to related plant species that are native in the expansion zone.

Plants are usually attacked by a wide variety of aboveground and belowground natural enemies (van der Putten *et al.*, 2001). It is well established that invasive exotic plants are less exposed to aboveground and belowground control by natural enemies than related natives in the new range (Klironomos, 2002; Maron & Vila, 2001; Callaway *et al.*, 2004; Reinhart *et al.*, 2003; Mitchell & Power, 2003; van der Putten *et al.*, 2005). However, phylogenetically controlled empirical evidence of exotic plant control by natural enemies is elusive (van Grunsven *et al.*, 2007; Agrawal *et al.*, 2005). Here, we compare range-expanding invasive plants of intercontinental origin and intracontinental range-expanding species with congeneric native plant species, all co-occurring in a riverine area. Aboveground, we exposed range expanding exotic plants of inter and intra-continental origin and congeneric native species to non-coevolved naïve polyphagous herbivores, as well as to cosmopolitan polyphagous herbivores. In the same experiment, we exposed all plants to a general soil community from the invaded range and compared their plant-soil feedback responses (Bever *et al.*, 1997). We tested the hypothesis that the plants would not differ in their response to the polyphagous shoot herbivores, as all plants had equal familiarity with them, but that both the inter- and intracontinental range-expanding species would develop less negative soil feedback than the related natives.

Methods

Floristic data were analyzed to identify exotic plant species in riparian areas in the Netherlands, which all have become well established in the 20th century. We surveyed plants with a strong increase in abundance over the past few decades with congeneric relatives in the same habitat. We obtained seedlings of a selection of three intracontinental range expanders, three species that originated from other continents and naturalized in southern Europe prior to their northward range expansion, and

nine natives (Table S1). Three extra native plant species were included to test the sensitivity of our phylogenetic comparison for species-specific effects. Soil samples were collected from Millingerwaard, inoculated into sterilized sandy loam soil, placed in 4-L pots and planted with 4 individuals of one species pot^{-1}. After 8 weeks in a greenhouse, the plants were harvested and the soils were used for a second growth experiment in order to measure plant-soil feedback effects (Bever *et al.*, 1997; van der Putten *et al.*, 2007a). In that second stage, each plant species was grown in own soil (previously containing individuals of the same species) and control soil (a mixture of soil from all other plant species, excluding species from the same genus). After 7 weeks, we placed all pots individually in cages and added aboveground herbivores to half of the pots that had been assigned to the herbivory treatment at the start of the experiment (n = 5). We used 5-day-old first instar locust nymphs of the African desert locust, *Schistocerca gregaria* (Forskål) which is highly polyphagous throughout all stages of its development and is non-coevolved with any of the tested plant species. Also *Myzus persicae* (Homoptera; Aphididae), the green peach aphid, a highly polyphagous herbivore, was used which has a cosmopolitan distribution. Three weeks after adding the herbivores, all plants were harvested, dried, weighed, and analyzed.

Results

Opposite to our hypothesis, aboveground herbivory influenced plant biomass of range expanding species differently from the natives (plant origin x herbivory interaction: $F_{1,108}$ = 4.58; P = 0.035; Figure 1a). Herbivores caused significant biomass loss to native plants (the species mean proportional biomass reduction was -38.7% and differed from zero: t = -2.98, d.f. = 8, P = 0.017; Figure 2a), whereas the effect of herbivory on the range-expanding species was much smaller and not significantly different from zero (effect size -17.3%: t = -1.69, d.f. = 5, P = 0.151; Figure 2a).

Although the range expanding species overall had more shoot biomass than the native species (P < 0.0001), locust survival was significantly lower on the range expanding than on the native species ($F_{2,52}$ = 9.57, P = 0.0003 after Post-hoc Tukey; Figure 3a). Aphid numbers, on the other hand were not significantly affected by host plant origin (n = 15, H = 0.897, P = 0.639; Figure 3b). The negative effect of the range-expanding plants on the locusts could not be explained by two general indicators of food quality, C/N-ratio and N content of the foliage (P = 0.197 and P = 0.597 respectively). Interestingly, the levels of phenolic compounds in the foliage were higher in range-expanding plants with herbivory than in range-expanding plants without herbivory and in the native plants with and without herbivory (interaction effect $F_{1,103}$ = 13.07; P = 0.0005; Figure S1).

This indicates that range expanding plants were better than natives in inducing general defenses against non-coevolved shoot herbivores. The intercontinental range expanders were slightly less negatively affected by herbivory than the intracontinental range expanders (range expander origin x herbivory: $F_{1,44}$ =

4.25, $P = 0.045$; Figure S2a). Nevertheless, the three intracontinental range expanders suffered significantly less from shoot herbivory than the congeneric natives (origin x herbivory $F_{1,52} = 6.45$; $P = 0.014$). *Bidens* was the only genus to show contrasting effects between native species within a genus (Figure S3a).
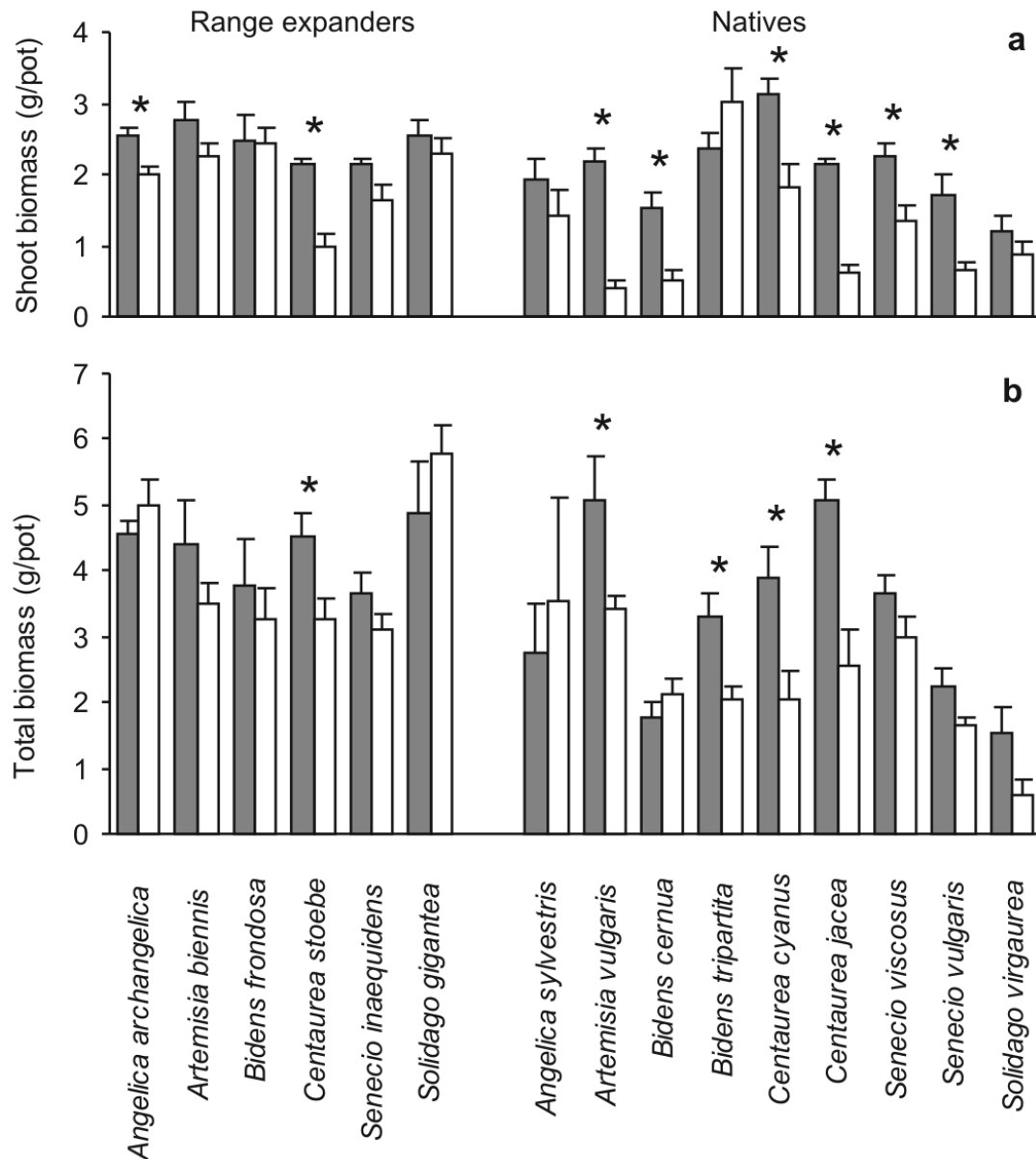

Figure 1 Biomass of range-expanding exotic and related native plants as influenced by non-coevolved and cosmopolitan polyphagous shoot herbivores and by soil feedback. Upper panel (a): shoot biomass (mean dry weight ± s.e.) of range-expanding exotic and congeneric native plants without herbivory (grey bars) and plants exposed to aboveground herbivory by the desert locust (*Schistocerca gregaria*) and the green peach aphid (*Myzus persicae*) (white bars) show that most plants experienced a significant biomass loss during three weeks of exposure, but that biomass loss due to herbivory was severest on native plants. Lower panel (b): total biomass (mean dry weight ± s.e.m.) on control soil (grey bars) and own soil (white bars) shows that natives are reduced more than range-expanding exotic species on own as compared to control soil. Bars show back-transformed means of log-transformed data. In both panels, an asterisk above a pair of bars indicates statistically significant effects of treatment within plant species (t-test, $P < 0.05$).
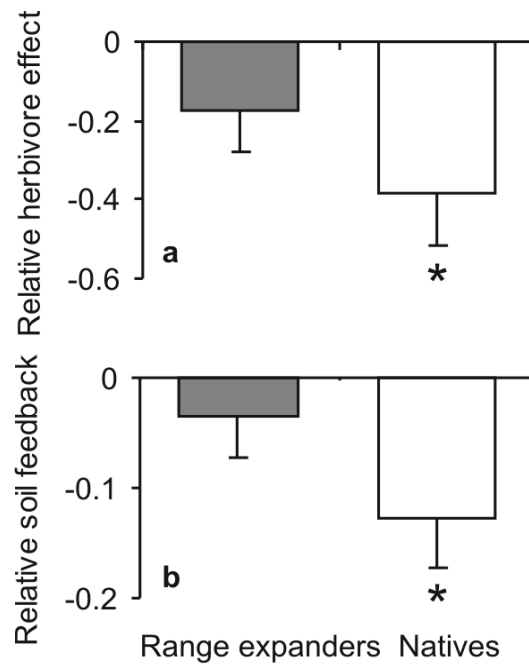
Figure 2 Average responses of range-expanding exotic plants (grey bars; n = 6 species averages) and related native plants (white bars; n = 9 species averages) to herbivory by non-coevolved and cosmopolitan polyphagous shoot herbivores and by soil feedback. Upper panel (a): Relative effects of aboveground herbivory by the desert locust (*Schistocerca gregaria*) and the green peach aphid (*Myzus persicae*) on shoot biomass (mean dry weight ± s.e.). Lower panel (b): The feedback effect of the soil community to total biomass production. Native species on average experienced significant negative soil feedback (indicated by asterisks; $P < 0.05$), whereas exotic range-expanding plants did not differ from a neutral response ($P > 0.05$). Panel (b) shows back-transformed means of log-transformed data.

Native plant species also suffered more from belowground biotic interactions in their own soil compared to control soil than range-expanding plants (plant origin x soil interaction: $F_{1,112} = 4.16$, $P < 0.043$; Figure 1b). The native species experienced significantly negative soil feedback (-12.8%, difference from zero: $t = -2.52$, d.f. = 8, $P = 0.036$; Figure 2b), whereas that of the range expanders was much smaller and not different from a neutral effect (-3.7%, difference from zero: $t = -0.96$, d.f. = 5, $P = 0.381$; Figure 2b). The performance in own versus control soil did not differ between the intra- and intercontinental range expanders (range-expander origin, soil and the interaction between range-expander origin x soil are: $F_{1,46} = 0.41$, $P = 0.526$; $F_{1,46} = 2.39$, $P = 0.129$ and $F_{1,46} = 0.84$, $P = 0.363$; Figure S2b). As observed for aboveground herbivores, a contrasting effect between native species within genus was observed for *Bidens* only (Figure S3b).

Across the herbivory and soil-feedback treatments, in 14 out of 18 within-genus comparisons the biomass reduction of the natives was stronger than of the range-expanders (non-parametric Sign Test, $M = -5$, $P = 0.031$; see Supplementary Information). However, above- and belowground biotic interactions did not vary in concert with each other; Spearman's rank order correlation of the shoot herbivore and soil feedback effects on species within sets of native and range-expanding plant
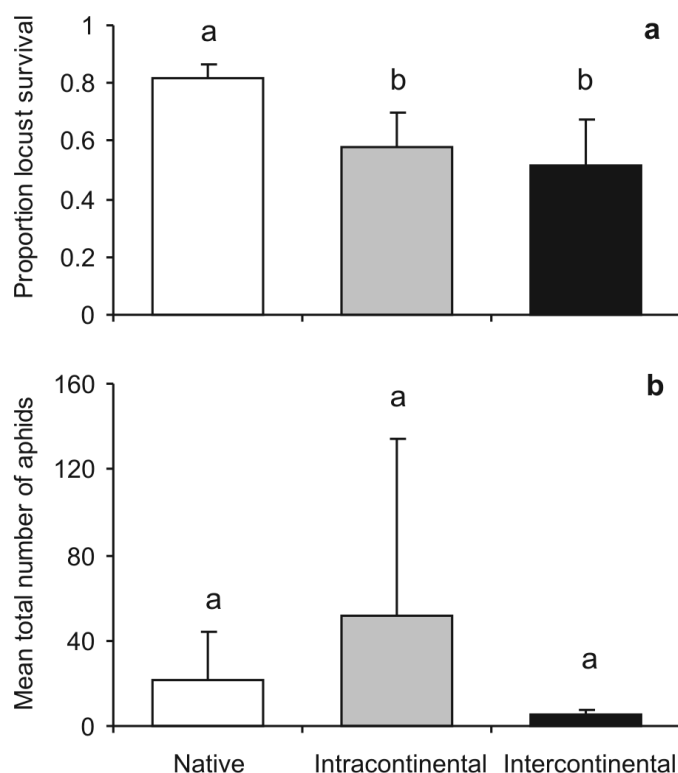
Figure 3 Performance of non-coevolved and cosmopolitan polyphagous shoot herbivores on native plant species and range-expanding species of intra- and intercontinental exotic origin. Upper panel (a): The proportion of survival (back-transformed means ± s.e. from arcsine data) of the naive generalist herbivore *Schistocerca gregaria*, that did not have any previous experience with any of the plant species used, on native (white bars), intracontinental range expanders (grey bars) and intercontinental range expanders (black bars) shows an on average lower survival on range expanders from both origins relative to native host plant species. Letters indicate significant differences between bars. Lower panel (b): Mean total numbers (± s.d.) of the generalist aphid *Myzus persicae* after 3 weeks feeding assay demonstrate that the on average population increase is independent of the origin of host plants; native (white bars), intracontinental (grey bars) and intercontinental (black bars) respectively.

species were not significant ($P = 0.865$ and $P = 0.329$ respectively; see Supplementary Information), we conclude that although range-expanding plants were less sensitive to shoot herbivory and negative soil feedback than natives, the magnitude of the above- and belowground effects did not necessarily vary in the same order.

Discussion

Our results provide new evidence that plants which are successful in range expansion towards higher latitudes interact more differently with shoot herbivores than congeneric plant species that are native to the invaded range. Although all plant species were equally novel to the desert locust, the locusts experienced reduced survival on these successful range expanders, but not on these related native plants. On the other hand, the cosmopolitan aphid was not influenced

differentially by plant origin. Our hypothesis predicted no differences; however, the shoot herbivores reduced the biomass of these range expanding plants less than that of these related native plant species. The negative soil feedback of these native plants as compared to these range expanders was more in line with our hypothesis. Thus far, studies on enemy exposure to exotic invasive weeds have usually focused on enemies from the invaded range, or on invasive enemies (Parker *et al.*, 2006). Our results suggest that the plant species successfully expanding their range towards higher latitudinal riparian areas possess superior defense traits when compared to related native species. In this respect, these successful range expanders have similarities with invasive exotic plants (Agrawal *et al.*, 2005), which also are superior in short-term resource acquisition (Funk & Vitousek, 2007), although there was no correlation between the strengths of above- and belowground enemy effects.

Thus far, most attention has focused on the uncoupling of food chain interactions due to regional climate warming (Menendez *et al.*, 2008; Davis *et al.*, 1998; Both & Visser, 2001). Here we show that some successful range-expanding riparian plant species (Tamis *et al.*, 2005) have less aboveground and belowground enemy impacts, even when exposed to non-coevolved and cosmopolitan polyphagous aboveground herbivores. Thus, these successful range expanders differed in defense trait characteristics from these congeneric natives. Our sampling strategy was focused on successful range expanders into northern riparian habitats. Future studies should also explore other habitats, as well as less successful range expanders in order to test whether, for example, trees and dry land plant species show similar responses. Poor range shift capacity has been predicted to result in a loss of diversity (Warren *et al.*, 2001; Thomas *et al.*, 2004a). However, the prediction of consequences of climate warming and other changes that result in range expansion require inputs from different fields in ecology (Guisan & Thuiller, 2005). Our results suggest that successful range-expanding plant species may include species with invasive properties, which is crucial information for the future conservation of biodiversity in temperate and northern latitudes.

Acknowledgements

Supplementary information

Plant species, seeds, soil and aboveground herbivores
We set out to compare exotic range-expanding and related native species according to the following criteria; the range-expanding plants have established in the Netherlands in the 20th century, they increased in grid cell abundance in the last decades of the 20th century, they have related native species in the same genus and they all occur in the same habitat. This information was derived from the National Standard List of the Dutch flora (Tamis, 2005) using square kilometer frequency records collected before 1950, between 1975-1987 and between 1988-1999. In order to calculate the national frequency of a plant species, we first calculated the sum of the proportional presence in all the 25 regions of the Netherlands. This proportional presence is calculated by the regional presence of a species (F_E) times its frequency in surveyed kilometer cells *(W_E)*. The national presence of a plant species in the Netherlands *(P_{Neth})* is calculated by multiplying the sum in all regions by 1000 divided by the total number of kilometer cells in the Netherlands *(A_{Neth})*. This national presence of a species is expressed as a permillage of all kilometer grid cells (Tamis, 2005).

$$P_{Neth =} \frac{1000}{A_{Neth}} \times \sum_{E=1}^{E=25} F_E \times W_E$$

Plants were considered successful range expanders if they were first recorded throughout in the twentieth century and showed a 10-fold increase in frequency in the last decade (nineteen nineties) when compared to the first half of the twentieth century. Based on the above criteria we chose to census range expanders and congeneric natives from the same riverine habitat of the Gelderse Poort region. The floristic database search yielded 17 successful range expanders from this habitat type. From these, we included in the experiment all species (six, from six different genera) that had a native congeneric species occurring in the same habitat and for which we could obtain and successfully germinate seeds. For each exotic range expander we included in the study one (three genera) or two (three genera) native species from the same genus (Table S1). All species belong to the family *Asteraceae*, except for the genus *Angelica (Apiaceae)*.

Seeds were collected from the field or, in some cases, purchased through a specialized seed supplier who collects seeds from local plant populations. All seeds were surface sterilized by a 1% hyperchloride solution and germinated on glass beads supplied with demineralized water at a 10-20°C, 10-14 hrs night-day regime for early summer species and a 15-25°C, 8-16 hrs night-day regime for late summer species. In order to synchronize the ontogeny, the seedlings were placed at 4°C with continuous illumination until transplantation. After transplantation, dead seedlings were replaced until the third week of the experiment.

Table S1 Origin, climate characteristics and frequency of occurrence of 6 range expanding exotic and 9 related native plant species that have been used in the present study. Range expanding species originate from either Eurasia or other continents; all species entered the Netherlands through range expansion. The original range of distribution of each species (4th column) is matched with the climate conditions (3rd column) within this range according to the modified Köppen-Geiger climate classification (Kottek *et al.*, 2006). The 5th and 6th columns show species frequencies of occurrence in the Netherlands before 1950 and at the end of the 20th century, respectively. These frequencies indicate the amount of grid cells across the Netherlands occupied by that specific plant species before 1950 and between 1988 and 2000, expressed on a per mil basis. In the last column, the percent change in frequency is based on the number of grid cells in the Netherlands where the species have been observed (see Supplementary methods above for calculation) after 1988, when temperatures started to rise (Tamis *et al.*, 2005). When considering species as replicates, the change in frequency is significantly higher for exotic range expanding species than for native species (one-way ANOVA; $F_{1,13}$ = 23.48, $P < 0.001$).

Species	Continental origin	Climate range of origin	Geographical origin	Freq. (‰) < 1950	Freq. (‰) 1988-2000	Change (%) 1950-2000
Angelica archangelica	Eurasian	Humid continental	NE-Europe	9	49	437
Angelica sylvertris	Eurasian	Marine west coast	NL	474	460	-2.96
Artemisia biennis	Eurasian	Subarctic-Humid continental	N-Asia	0.4	3	669
Artemisia vulgaris	Eurasian	Marine west coast	NL	739	792	41.9
Centaurea stoebe	Eurasian	Humid continental-Mediterranean	C-Europe	0.05	0.1	82.2
Centaurea cyanus	Eurasian	Marine west coast	NL	147	75	-49.3
Centaurea jacea	Eurasian	Marine west coast	NL	379	317	-16.5
Bidens frondosa	non-Eurasian	Humid continental-Subarctic-Mid latitude steppe	N-America	79	225	186
Bidens cernua	Eurasian	Marine west coast	NL	155	175	12.8
Bidens tripartita	Eurasian	Marine west coast	NL	360	345	-3.92
Senecio inaequidens	non-Eurasian	Humid subtropical	S-Africa	3	119	3634
Senecio viscosus	Eurasian	Marine west coast	NL	93	161	73.3
Senecio vulgaris	Eurasian	Marine west coast	NL	739	792	7.25
Solidago gigantea	non-Eurasian	Subarctic-Mid latitude steppe	N-America	36	193	442
Solidago virgaurea	Eurasian	Marine west coast	NL	27	16	-42.9

We collected soil from five randomly chosen sites in the Millingerwaard (the Netherlands; 51°87′ N, 6°01′ E), a nature reserve in the Gelderse Poort region where all range expanding and related native plant species co-occur. The soil samples were homogenized, as we were not interested in spatial variation in the field, and used as an inoculum and introduced into a sterilized sandy loam soil from Mossel, Planken Wambuis (52°06′ N, 5°75′ E). The soil sterilization was carried out by gamma radiation (25 kGray), which eliminated all soil biota (van der Putten *et al.*, 2007a).

As a naive herbivore, we choose the generalistic African desert locust *Schistocerca gregaria* (Forskål), because this species is highly polyphagous during its gregarious phase. This locust is not native to the Netherlands and is unlikely to share a co-evolutionary history with any of the plant species used, as it occurs in north-central Africa and Asia. The exclusive circumstance of the feeding naïveness of the locust towards all plant species, enabled us to consider all plants, both the range expanding and the native species, as having defenses which are potentially novel to the herbivore. The nymphs were obtained from a gregarious rearing on grasses of the Laboratory of Entomology of Wageningen University, the Netherlands. As a cosmopolitan generalist herbivore, we selected *Myzus persicae* (Homoptera;

Aphididae), the green peach aphid, which was obtained from a culture from Wageningen University. It is highly polyphagous and feeds on a wide variety of host plant families.

Experimental setup

Phase I: soil conditioning. One hundred and fifty 4-L pots were filled with a 5:1 mixture of sterilized soil and inoculum soil collected from Millingerwaard. We established 10 replicate pots of each plant species (6 range expanders and 9 natives). Each pot received 4 seedlings and the experiment was carried out in a greenhouse under controlled conditions (60% RH, day: 21 ± 2°C; night 16 ± 2°C). Additional light was provided by metal halide lamps (225 µmol $^{-1}$ m^{-2} PAR) to ensure a minimum light intensity during 14 hr daytime. Plants were provided with demineralized water every second day to compensate for water uptake and evapotranspiration. Every week, initial soil moisture level was reset by weighing. In order to prevent plants from nutrient depletion, Hoagland solution was added at a rate of 25 ml of 0.5 strength week^{-1}, which is a dosage that does not prevent the establishment of arbuscular mycorrhizal fungi (van der Putten *et al.*, 2007a). After 8 weeks of growth, the plants were harvested and the conditioned soils were used for a second growth phase to test the plant-soil feedback effect and the effect of aboveground herbivory.

Phase IIa: soil feedback. The conditioned soil from every pot in phase I of the growth experiment was split into two halves. One half was placed in a 1.3-L pot to be called 'own' soil. The other half was used to create a pot with control soil. The control soil of every plant species contained soil conditioned by all other plant species, excluding plants from the same genus. We established five replicates with own and ten with control soils: each replicate was made from a separate replicate from the soil conditioning phase. Five of the ten pots with control soils were assigned randomly to a shoot herbivory treatment (further described below at Phase IIb). We planted 3 seedlings per pot. Water, light and nutrient conditions were supplied as in phase I, except that 10 ml of 0.5 Hoagland solution was added on a weekly basis. This reduced and more concentrated rate was necessary, because the pots were smaller, there were fewer plants and there was less evaporation from the soil surface. After week 10, all roots and shoots were harvested, air-dried at 70°C for 48 hrs and weighed as total root and shoot biomass per pot. Soil feedback was calculated using total (shoot and root) dry biomass. Soil feedback was calculated for each replicate separately as: (total biomass own soil – total biomass control soil) / (total biomass control soil). A negative feedback indicates net pathogenic activity, whereas a positive feedback indicates net symbiotic activity, whereas a neutral feedback indicates that pathogens and symbionts are either not active, or that they neutralize the effects on each other (van der Putten *et al.*, 2007a).

Phase IIb: shoot herbivory. In the 7th week, the five replicates of the control plants that had been assigned randomly to the shoot herbivory treatment at the start of the experiment were exposed to the locusts, which were added at a rate of 3.pot^{-1}. The African desert locusts (average weight = 0.0858 g, n = 79) and the aphids were

prevented to escape by placing all pots (including those from the soil feedback experiment) individually in spherical nets (Ø 25 cm, height 1.5 m). Before the start of the treatment the locust nymphs were starved for 24 hrs. Subsequently, they were allowed to feed for 3 consecutive weeks until harvest. Once per week, locust survival was determined. The first cohorts of the aphids were reared on white radish (*Raphanus sativus*) in transparent boxes (40 cm x 50 cm x 65 cm) which were stored in a climate room with conditions of 21°C, a 14-hr light / 10-hr dark period and 60% RH. We started with 8 maternal lines which were mixed in the last growth cohort to ensure sufficient genetic diversity before being transferred to the experiment. From the rearing only apterous adults with similar size were selected. Each replicate from each plant species received 5 individuals. After 3 weeks of feeding we counted total number of aphids per replicate pot.

All phase II shoot herbivory and soil feedback pots were completely randomized in the greenhouse. Plants exposed to herbivory were harvested at the same time as the plants exposed to soil feedback, after week 10 (described above), and we analyzed herbivore effects on shoot biomass. Relative herbivory effects were calculated as (shoot biomass with herbivores – shoot biomass without herbivores) / (shoot biomass without herbivores). We also determined locust survival and aphid population growth (see above).

Chemical analyses
Shoot tissue C and N, as well as levels of phenolic compounds, which are general plant defensive chemicals (Hunter & Forkner, 1999) were determined and insects were counted. Whole dry shoots were used to analyze total phenolic content following a modified Folin-Denis protocol (Waterman & Mole, 1994). Dry plant material was ground and 0.025 g was weighed into a test tube with 5 ml of 1.2 M HCL in 50% aqueous methanol, heated for 2 hrs at 90°C and centrifuged at 6000 rpm for 10 min. Folin–Denis reagents (0.2 ml) was mixed with 0.2 ml supernatant and 1.0 ml Na_2CO_3. After 30 min and centrifuging at 14000 rpm for 5 min absorption at 750 nm was measured to determine total phenol content to be calculated as percentage of the dry weight. In addition, total C and N contents of the whole shoots were determined by catalytic oxidation and gas chromatography (Nieuwenhuize *et al.*, 1994).

Statistical analyses
Effects of herbivores or soil pre-treatment on plant biomass were analyzed separately in the fixed effects ANOVA model: $Y_{ijkl} = \mu + O_i + T_j + SO_{k(i)} + T*O_{ij} + T*SO_{jk(i)} + \varepsilon$; where Y_{ijkl} is the shoot biomass (herbivory tests) or the log total (ln(biomass+1)-transformation; soil feedback tests) for the lth plant from the kth species in the jth treatment; T is the treatment effect, O is the origin effect and S is the species effect, where species are nested within origins. All variables were considered fixed effects. Species was considered fixed because our approach was to census the available exotics (that meet our criteria for successful range expansion) plus their genus-

matched natives from a specific riverine habitat type; our procedure for species selection (see above) did not result in a random sample of natives and exotics. Note that the decision to consider species as a fixed nested effect results in statistical testing of all model terms over the model residual error (Neter *et al.*, 1996) and as a consequence statistical inference is limited to the set of species that are included in the study, although and as argued above, the species represented a selection as complete as possible for such riverine ecosystems.

Of primary interest is the treatment x origin interaction, indicating whether treatment effects differ between native and range expanding species. Similar models were used to test for differences between native and range-expanding species in nitrogen content, C/N ratio, levels of phenolic compounds in the herbivore assay. To analyze origin effects on locust survival, aphid numbers were added as a covariate. These analyses were performed in SAS version 9.1 for Windows (proc MIXED, SAS Institute Inc., Cary, USA). Aphid scores did not meet standard assumptions for analysis of variance and we therefore analyzed the effect of plant origin (native, intracontinental and intercontinental) on aphid numbers using a nonparametric Kruskal-Wallis test based on species mean values. Additional t-tests were performed for each species individually to assess significance of the treatment effect at the species level, and we also used t-tests (based on species mean values) to test whether the soil feedback effect and the proportional herbivory effect of the natives and range expanders differed significantly from zero. To improve normality of residuals and homogeneity of variances among groups defined by the statistical models, plant biomass for soil-feedback analysis was ln-transformed, shoot phenolic content was square root-transformed, shoot N content was square rooted ln-transformed and locust survival was arcsine transformed prior to analysis. For the analysis on species frequencies the values were square rooted before log transformation. In order to test for a relationship between effects of shoot herbivory and soil feedback a Spearman rank order correlation was performed with the species as replicate units. We did not find a significant correlation, neither for the range expanders (n = 6; R^2 = 0.236, P = 0.329), nor for the natives (n = 9; R^2 = 0.004, P = 0.865). Therefore, we concluded that plants that although range-expanding plants were less sensitive to shoot herbivory and negative soil feedback than natives, the magnitude of the above- and belowground effects did not necessarily vary in the same order.

Treatment effect sizes for individual species and within-genus comparisons between range expander and native species
The overall analysis of the herbivory dataset and the soil feedback dataset revealed significant treatment-by-origin interactions, with natives suffering more biomass loss than exotics due to the herbivory and the 'own soil' treatments. In order to assess the generality of this pattern across the different species that were included in the study, Figures S4 and S5 show plots of the raw data for each species. For each species the effect size is given as the difference between the means of the two treatment levels, and p values are from t-tests of treatment effect within each species separately. The

treatment effect is more often significant in native species than in exotic range expanding species. The effect of herbivory is significant in 6 out of 9 natives versus 2 out of 6 exotics (Figure S4) and the effect of soil feedback is significant in 4 out of 9 natives versus 1 out of 6 exotics (Figure S5). Within a genus, the biomass reduction caused by the treatment is typically stronger in the native than in the exotic congener. Both herbivory and soil feedback give a stronger biomass reduction (negative effect size) to the native congener in 7 out of 9 within-genus comparisons (Figures S4 and S5).
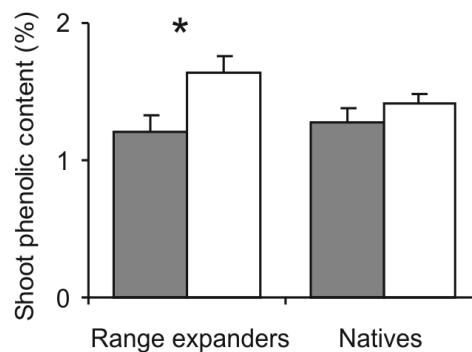

Figure S1 Phenolic content of range-expanding and native plant species with and without herbivory. Bars show average phenolic content (percentage/g ± s.e.) in plant shoot with herbivory (white bars) and controls (grey bars) for range-expanding (n = 6 species averages) and native (n = 9 species averages) plant species. Only range expanding plants exhibit significant (∗ P < 0.05) higher phenolic contents when exposed to herbivory. Back-transformed data of square root transformed data are shown.
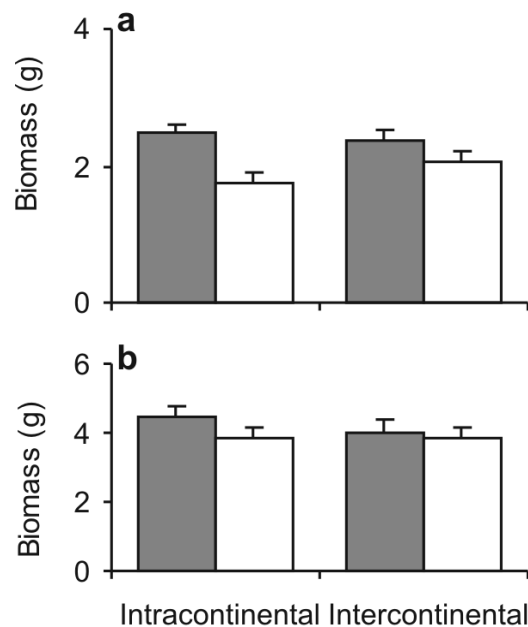
Figure S2 Results of soil feedback and herbivory between intracontinental (Eurasian) and intercontinental (non-Eurasian) range-expanding plant species. Upper panel (a): Shoot biomass (mean dry weight ± s.e.) without herbivores (grey bars) and with herbivores (white bars) shows that intercontinental range-expanding species were slightly less negative affected by herbivory than the intracontinental range expanders (range expander origin x herbivory: $F_{1,44}$ = 4.25, P = 0.045). Lower panel (b): Total biomass (mean dry weight ± s.e.) on control soil (grey bars) and own soil (white bars) shows that soil feedback was not different (P > 0.05) between inter- and intracontinental range expanders.

Over the two experiments, in 14 out of 18 within-genus comparisons the treatment response was stronger in the native species. Using a Sign Test, this is significantly more often than would be expected by chance ($M = -5$, $P = 0.031$). The non-parametric Sign Test is considered an insensitive, low-power test. Thus, the significant result provides strong evidence that the main results from the overall analysis, namely that natives respond more strongly to the treatments than exotics, hold very generally across the species and the genera that were included in the present study.
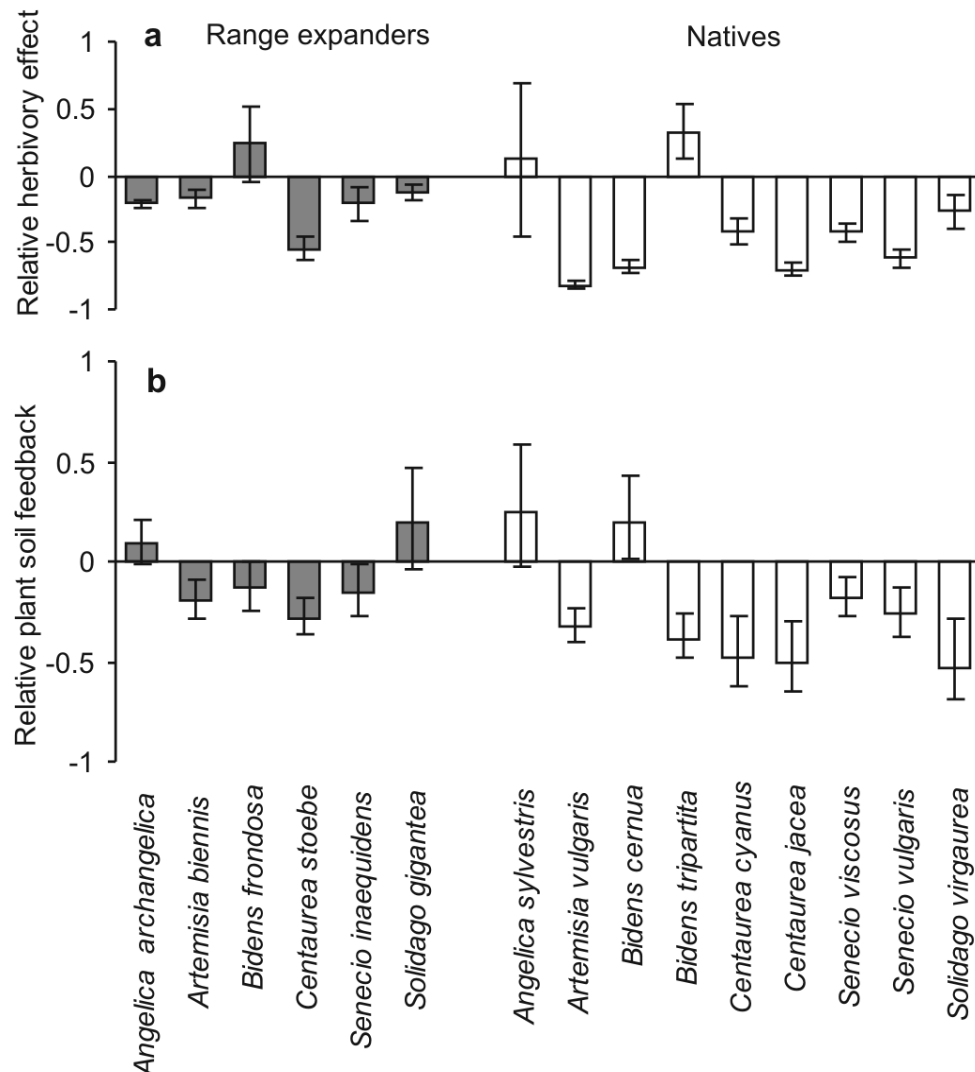
Figure S3 Individual proportional responses of the range expanding and native plant species to a naïve aboveground herbivore and soil feedback. Upper panel (a): Relative herbivore effect on range expander (grey bars) and native (white bars) shoot biomass ((shoot biomass with herbivores − shoot biomass without herbivores) / (shoot biomass without herbivores) ± s.e.) by *Schistocerca gregaria* and *Myzus persicae* reveals that there was individual variation among plant species, but that the overall impact on range-expanding plant species was lower than on native plant species. Lower panel (b): Relative plant soil feedback (back-transformed means of log-transformed data of ((total biomass own soil − total biomass control soil) / (total biomass control soil) ± s.e.) of range-expanding (grey bars) and native (white bars) species reveals variation among species, but an overall stronger negative impact of soil feedback on native than on range-expanding plant species.
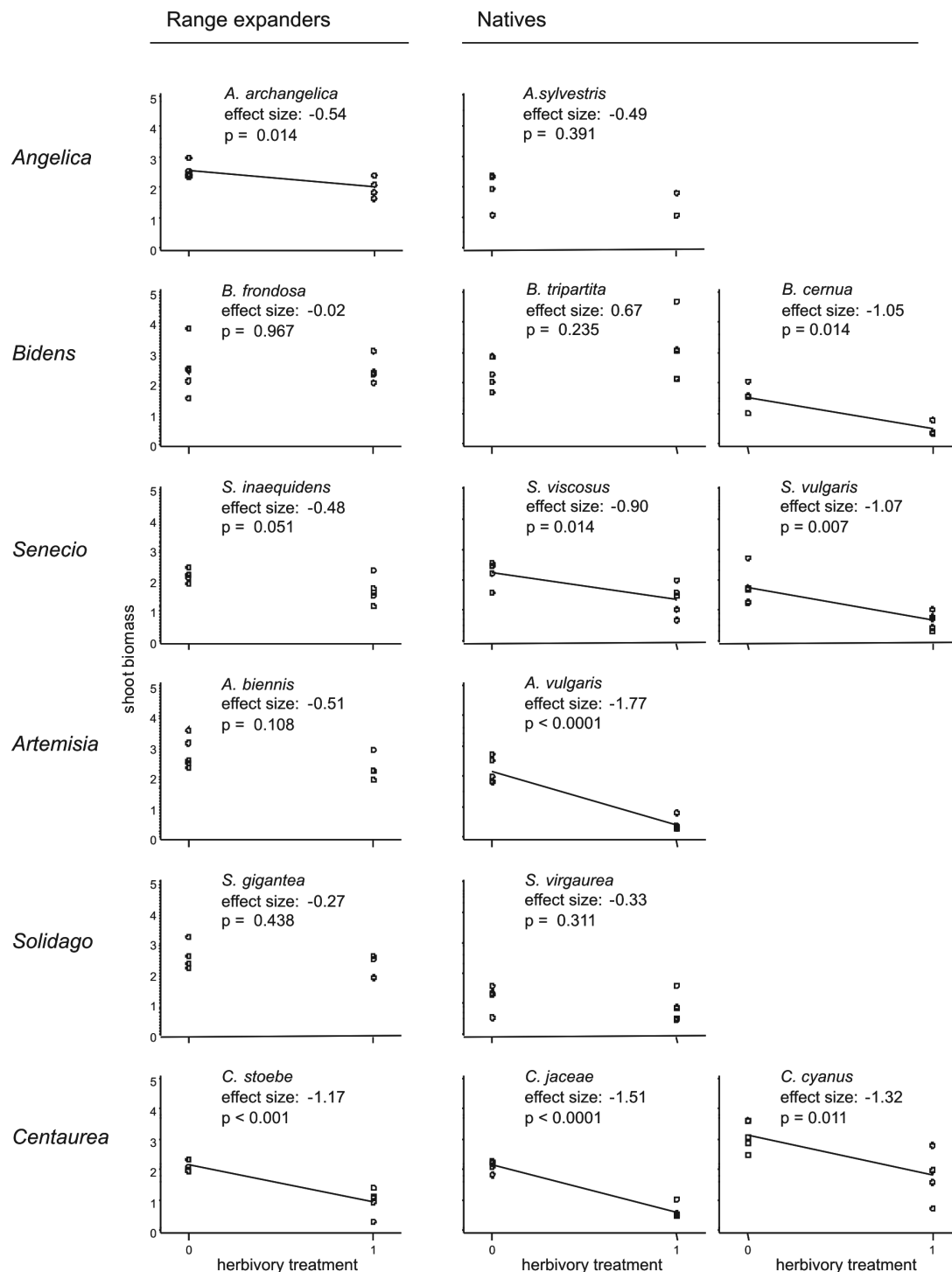
Figure S4 Plots of the raw data of the herbivore effects on each plant species. Herbivore effects on each plant species, arranged by genus (rows). Left panels are range-expanding species; middle and right panels are congeneric native species (the second native species was added to three genera in order to compare within genus effects between native congeners). Herbivory treatments: 0, without herbivores; 1, with herbivores. For each plant species (left the range expander, middle and right the congeneric native species; in three genera there were two congeneric natives) the effect size is given as the difference between the means of the two treatment levels, and *P* values are from t-tests of treatment effect within each plant species.
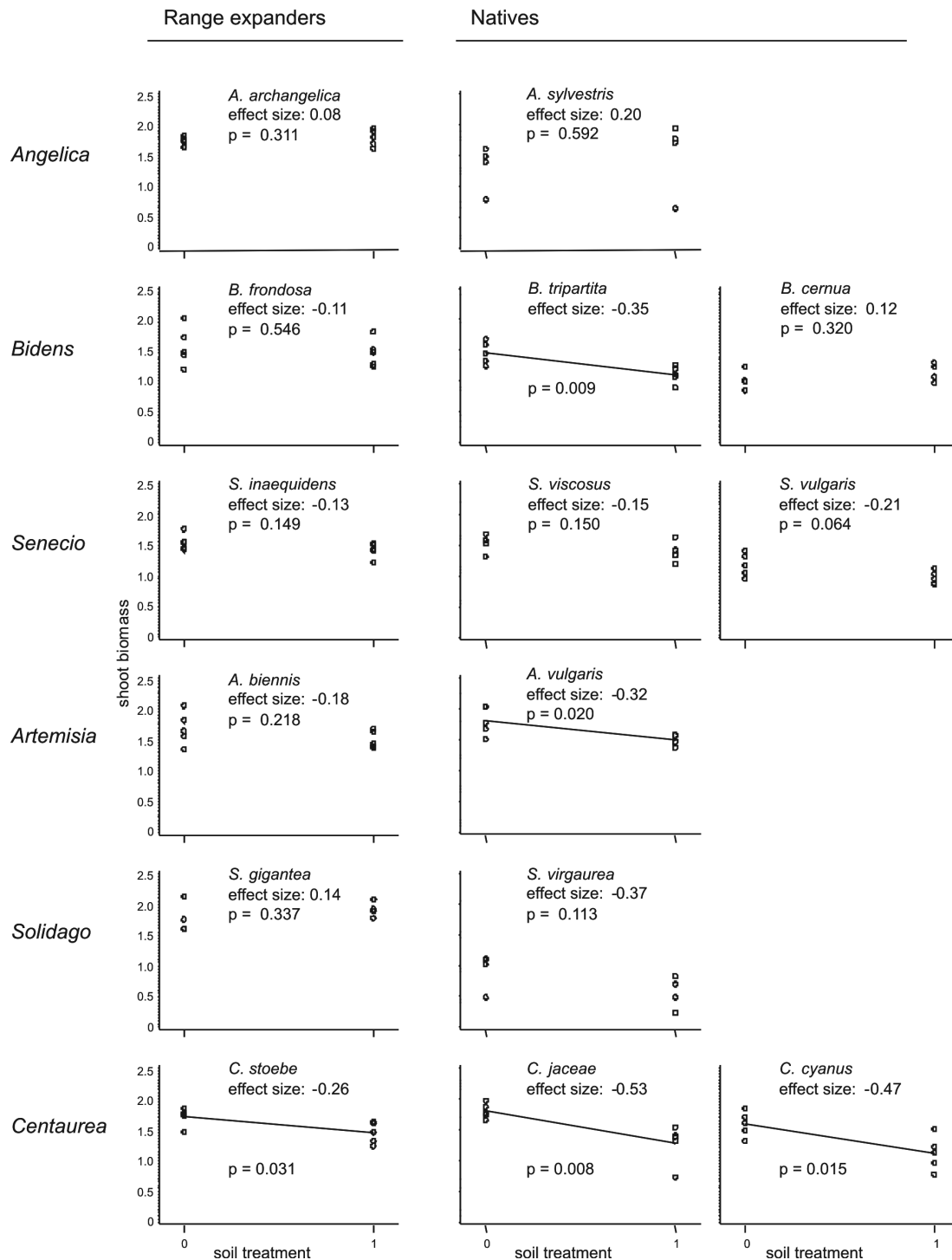
Figure S5 Plots of the raw data of the soil feedback effects on each plant species. Soil feedback effects on each plant species, arranged by genus (rows). Left panels are range-expanding species; middle and right panels are congeneric native species (the second native species was added to three genera in order to compare within genus effects between native congeners). Soil treatments: 0, control soil; 1, own soil. Total plant biomass scores are after ln(biomass + 1) transformation. For each plant species (left the range expander, middle and right the congeneric native species; in three genera there were two congeneric natives) the effect size is given as the difference between the means of the two treatment levels, and *P* values are from t-tests of treatment effect within each plant species.

Chapter 3

ADDITIVE EFFECTS OF ABOVEGROUND POLYPHAGOUS HERBIVORES AND SOIL FEEDBACK IN NATIVE AND RANGE-EXPANDING EXOTIC PLANTS

Elly Morriën, Tim Engelkes & Wim H. van der Putten

Abstract

Plant biomass and plant abundance can be controlled by aboveground and belowground natural enemies. However, little is known about how the aboveground and belowground enemy effects may add up. We exposed fifteen plant species to aboveground polyphagous insect herbivores and feedback effects from the soil community alone, as well as in combination. We envisaged three possibilities: additive, synergistic, or antagonistic effects of the aboveground and belowground enemies on plant biomass. In our analysis, we included native and phylogenetically related range-expanding exotic plant species, because exotic plants on average are less sensitive to aboveground herbivores and soil feedback than related natives. Thus, we examined if lower sensitivity of exotic plant species to enemies also alters aboveground-belowground interactions. In a greenhouse experiment, we exposed six exotic and nine native plant species to feedback from their own soil communities, aboveground herbivory by polyphagous insects, or a combination of soil feedback and aboveground insects and compared shoot and root biomass to control plants without aboveground and belowground enemies. We observed that for both native and range expanding exotic plant species effects of insect herbivory aboveground and soil feedback added up linearly, instead of enforcing or counteracting each other. However, there was no correlation between the strength of aboveground herbivory and soil feedback. We conclude that effects of polyphagous aboveground herbivorous insects and soil feedback add up both in the case of native and related range-expanding exotic plant species, but that aboveground herbivory effects may not necessarily predict the strengths of soil feedback effects.

Introduction

In many studies it has been considered how aboveground or belowground natural enemies may control biomass production and abundance of native or invasive exotic plant species (Bardgett & Wardle, 2010). However, there are very few examples of examining how plant control effects by aboveground and belowground enemies may interact and whether these interaction effects may differ between native and invasive exotic plant species. Here we show how aboveground and belowground plant enemies alone or in combination influence biomass production of native and range expanding exotic plant species. Thus far, most studies on aboveground-belowground interactions have focused on influences of aboveground and belowground herbivores, pathogens or mutualists on each other (e.g. Masters *et al.*, 1993; Bennett & Bever, 2007) or on interactions between aboveground herbivores and belowground decomposers (Bardgett & Wardle, 2003). A number of studies have tested how aboveground and belowground herbivores may influence the composition of plant communities (Brown & Gange, 1989; Schädler *et al.*, 2004; van Ruijven *et al.*, 2005). Few studies, however, have considered how plant exposure to both aboveground and belowground herbivores and pathogens may influence individual plants (Maron, 1998).

The possible outcomes of combined effects of aboveground and belowground plant enemies on plant performance may be that the effects add up linearly (additive effects), enhance each other so that the combined effects are stronger than the two individual effects together (synergistic effects), or counteract each other so that the combined effects are weaker than the individual effects together (antagonistic effects). Such additive, synergistic, and antagonistic effects have been observed aboveground, as well as belowground. For example, in papaya (*Carica papaya*) and hoary cress (*Lepidium draba*) exposing plant individuals to two or more aboveground herbivore species resulted in additive effects (Fournier *et al.*, 2006; Puliafico *et al.*, 2008), whereas exposing the foredune grass *Ammophila arenaria* to a number of pathogenic soil fungi and root-feeding nematodes resulted in synergistic growth reduction effects (de Rooij-van der Goes, 1995). The interaction effects of plant enemies may depend on the number of interacting enemy species: effects of root-feeding nematode species on *A. arenaria* were additive when exposing plants to two species (Brinkman *et al.*, 2008), but antagonistic when testing effects of three nematode species (Brinkman *et al.*, 2005). As far as we know only Maron (1998) tested effects of aboveground and belowground herbivores simultaneously on individual plants. His study on bush lupine (*Lupinus arboreus*) showed additively negative effects of root-boring moth larvae and flower- and seed-feeding insects on the production of seed pods. This previous work leaves the question still wide open how plant individuals may respond to individual and combined effects of aboveground and belowground enemies.

In field studies, selective aboveground and belowground insecticides showed contrasting effects of chemical insect exclusion on plant community development.

Aboveground insecticides enhanced vegetation succession, whereas succession was slowed down by adding belowground insecticides (Brown & Gange, 1989; Schädler *et al.*, 2004). Moreover, aboveground insecticides enhanced the abundance of grasses while belowground insecticides enhanced forb cover in plant communities (Brown & Gange, 1989). The use of insecticides may be due to unaccounted side effects, for example by altering decomposition and nutrient mineralization. Nevertheless, experimental outdoor mesocosms with and without aboveground and belowground invertebrates had similar effects (van Ruijven *et al.*, 2005) as the field experiments of Brown & Gange (1989) with insecticides. Therefore, aboveground invertebrate herbivores can have opposite effects on plant community development compared to belowground invertebrate herbivores.

The field studies, however, reported combined effects of individual plant responses to aboveground and belowground herbivory and plant-plant interactions (De Deyn *et al.*, 2007). Hence, whereas these selective removal and addition studies revealed a profound role of aboveground and belowground invertebrate in processes influencing plant community composition, they provide limited insight in the underlying processes. Key questions that remain are for example how plant species respond to individual and combined effects of aboveground and belowground natural enemies, and how the sensitivity to aboveground enemies correlates with that to belowground enemies.

The main aim of the present study was to examine how individual plants respond to aboveground and belowground plant enemies and to their combined effects. Based on the previously reported work in aboveground or belowground subsystems, we expected effects varying from additive to synergistic, or even antagonistic. As field experiments suggested different responses of plant community composition to aboveground and belowground plant enemies (Brown & Gange, 1989; Schädler *et al.*, 2004; van Ruijven *et al.*, 2005), we expected that the strength of aboveground effects will not necessarily correlate with the strength of belowground effects. This may be due to defense responses in plants to aboveground herbivores differing from belowground herbivores, or root pathogens (Bezemer & van Dam, 2005; Kaplan *et al.*, 2008; van Dam, 2009).

We included fifteen plant species in our study. Nine plant species were natives and six were range expanding exotic plant species that are rapidly increasing in abundance in the study region (Tamis, 2005). Biomass production of the range expanders is less influenced by aboveground and belowground enemies than that of natives (Engelkes *et al.*, 2008), which is similar to cross-continental invasive plant species (Keane & Crawley, 2002; Klironomos, 2002; Mitchell & Power, 2003; Agrawal *et al.*, 2005; Zhang *et al.*, 2009). As little is known about the combined aboveground-belowground enemy effects to exotic plants (Agrawal *et al.*, 2005), we examined if individual responses of the exotic range expanders to aboveground and belowground enemies deviates from the responses of native plant species.

As aboveground enemies, we choose the generalist African desert locust (*Schistocerca gregaria*) and the green peach aphid (*Myzus persicae*), because they

represent important feeding types (a leaf chewer and a phloem feeder, respectively), and because they are highly polyphagous. Our choice for soil feedback is that this approach integrates effects of plant enemies, symbionts and decomposer organisms (Bever *et al.*, 1997). Most plant species tested thus far have shown negative soil feedback effects (Bever, 2003; Petermann *et al.*, 2008), indicating that effects of soil pathogens overrule those of symbionts and decomposers (Bever *et al.*, 1997). Whereas the range expanding exotic plant species may experience less control from aboveground or belowground enemies, we expected them to respond similarly to individual versus combined enemy effects.

Considering the many unknowns, we formulated three null-hypotheses assuming that aboveground and belowground enemy effects would (1) add up in a linear fashion, (2) that this type of addition does not differ between natives and exotic range expanders and (3) that for both native and exotic plants the strength of aboveground control by polyphagous insects will be indicative of the strength of belowground control by plant-soil feedback. We tested these hypotheses in a full factorial greenhouse experiment.

Methods

Selection of plant species, collection of seeds and soil
We used range expanding exotics and phylogenetically related native plant species all from the same habitat in order not to confound our comparison with other factors. We collected plant seeds and soil from the Millingerwaard (the Netherlands; 51°87′ N, 6°01′ E), a relatively nutrient rich nature reserve (due to regular river floods) in the Gelderse Poort region after having made a full overview of the range-expanding exotic plant species in the study area. Then, for each range-expanding plant species we selected a native plant species within the same genus based on similar ecology. The plant pairs used in our experiment were selected using four criteria. First the exotic range-expanding plants should have established in the Netherlands, north-western Europe, in the 20th century in order to be considered introduced recently. Second, they should have increased in national grid cell abundance in the last decades of the 20th century in order to have their expansion response correlated positively to climate warming (Tamis, 2005). Third, they should have related native species in the same genus for phylogenetic comparison, and fourth, all native and range-expanding plant species should occur in the same habitat, in order to perform the whole experiment using the same soil.

The underlying information on which we based our choices was derived from the National Standard List of the Dutch flora (Tamis, 2005). In this dataset the Netherlands is divided in grid cells of one square kilometre. Local plant counts determine whether a grid cell is occupied by a certain plant species or not. This abundance measure ranges from zero (no grid cells occupied) to ten (all grid cells occupied). To examine how grid cell abundance increased in the 20th century, data were analyzed between 1902-1950, 1975-1988 and 1988-2000. Based on the above four

criteria and the availability of seeds for producing seedlings, we were able to select six genera: five from the *Asteraceae* and one from the *Apiaceae* family. Three exotic plant species originated from Eurasia and the other three exotics originated from other continents. In three genera, we included two native plant species to be able to see how similar two natives within genus are in their response compared to the exotic range expander (Table 1).

Seeds were collected from the field or, in a few cases, purchased through a specialized seed supplier who collects seeds from local plant populations. All seeds were surface sterilized by a 1% hyper chlorite solution and germinated on glass beads supplied with demineralized water at a 10-20°C, 10-14 hrs night-day regime for early summer species and a 15-25°C, 8-16 hrs night-day regime for late summer species. In order to synchronize the ontogeny, germinated seedlings were placed at 4°C with continuous illumination until transplantation. After transplantation, dead seedlings were replaced until the third week of the experiment.

The soil samples were collected from five random sites in Millingerwaard, homogenized, as we were not interested in spatial variation in the field, and inoculated into a sterilized sandy loam soil from Mossel, Planken Wambuis (52°06′ N, 5°75′ E). The soil sterilization was carried out by gamma radiation (25 kGray), which eliminated all soil biota (van der Putten *et al.*, 2007a).

Table 1 Plant species used in the experiment. Species names in first column, species origin in second column, the original range of distribution in third column. The last three columns indicate Kilometer Frequency Classes (KFCs), a measure for the amount of occupied grid cells in the Netherlands (see detailed explanation in methods section). All information is according to the Dutch standard list (Tamis, 2005). *Centaurea stoebe* has very recently invaded in the Netherlands and therefore no frequency records are available for this plant species.

Species	Origin	Geographical origin	KFC 1920-1950	KFC 1975-1988	KFC 1988-2000
Angelica archangelica	exotic	North East-Europe	4	6	7
Angelica sylvestris	native	The Netherlands	9	9	9
Artemisia biennis	exotic	North Asia	0	2	5
Artemisia vulgaris	native	The Netherlands	9	9	9
Centaurea stoebe	exotic	Central-Europe	-	-	-
Centaurea cyanus	native*	The Netherlands*	9	8	7
Centaurea jacea	native	The Netherlands	9	9	9
Bidens frondosa	exotic	North-America	6	8	8
Bidens cernua	native	The Netherlands	8	8	8
Bidens tripartita	native	The Netherlands	9	9	9
Senecio inaequidens	exotic	South-Africa	0	6	8
Senecio viscosus	native	The Netherlands	7	8	8
Senecio vulgaris	native	The Netherlands	9	9	9
Solidago gigantea	exotic	North-America	5	7	8
Solidago virgaurea	native	The Netherlands	7	7	6

* *Centaurea cyanus* is recorded as being archeophyte in the Netherlands. This plant was imported in the Middle-Ages from the East-Mediterranean area as a crop to use in dye. Since this plant has occurred in the Netherlands for centuries, we treated this species as being native in our experiment.

Experimental setup

Phase I: soil conditioning. One hundred and fifty four liter pots were filled with a 5:1 mixture of sterilized soil and inoculum soil. We established ten replicate pots of each plant species (six exotics and nine natives). Each pot received four seedlings and the experiment was carried out in a greenhouse under controlled conditions (60% RH, day: 21 ± 2°C; night 16 ± 2°C). Additional light was provided by metal halide lamps (225 μmol PAR/m^2, where PAR is photosynthetically active radiation) to ensure a minimum light intensity during 14 hr daytime. Plants were provided with demineralized water every second day to compensate for water uptake and evapotranspiration. Every week, initial soil moisture level was reset by weighing. In order to prevent plants from nutrient depletion, Hoagland solution was added at a rate of 25 ml of 0.5 strength per week, which is a dosage that does not prevent the establishment of arbuscular mycorrhizal fungi (van der Putten *et al.*, 2007a). Initial soil nutrient conditions were similar in all pots in the conditioning phase. After 8 weeks of growth, the plants were harvested and the conditioned soils were used for a second growth phase to test the plant-soil feedback effect and the effect of aboveground herbivory.

   Phase II: soil feedback. The conditioned soil from every pot in phase I of the growth experiment was split into two halves. One half was placed in a 1.3 liter pot to be called 'own' soil. The other half was used to create control soils. The control soil of every plant species contained soil conditioned by all other plant species, excluding plants from the same genus. Because all controls shared soil from five genera, we assumed initial soil nutrient conditions to be similar for all control pots. We established ten replicates with own and ten with control soils: each replicate was made from a separate replicate from the soil conditioning phase. Five of the ten replicate pots with control soils were randomly assigned to an aboveground herbivory treatment. We planted three seedlings per pot. Water, light and nutrient conditions were supplied as in phase I, except that 10 ml of 0.5 Hoagland solution was added on a weekly basis because the pots were smaller, there were fewer plants and there was less water loss from the soil surface compared to the 4 liter pots. After week 10, all roots and shoots were harvested, air-dried at 70°C for 48 hrs and weighed as total root and shoot biomass per pot. Relative soil-feedback was calculated using total (shoot and root) dry biomass for each replicate separately as: (total biomass own soil – total biomass control soil) / (total biomass control soil) (van der Putten *et al.*, 2007a). At the end of the soil feedback phase we measured mineral nitrogen NO_3-N and NH_4-N in 0.01 M $CaCl_2$-extract (soil:extract 1:10) and available phosphorus also in 0.01 M $CaCl_2$-extract (soil:extract 1:10) colorimetrically using a Traacs 800 auto-analyzer (TechniCon Systems, Seal Analytical, Mequon, Wisconsin, USA) as a measure of nutrient depletion.

   Aboveground herbivory. In the 7th week, five replicates of the control pots and five replicates of the own pots that had been randomly assigned to the aboveground herbivory treatment were exposed to the locusts (3 individuals/pot) and the aphids (5 individuals/pot). The African desert locusts (average weight = 0.0858 g, n = 79) and

the aphids were prevented from escaping by placing all pots (including those from the soil feedback experiment) individually in spherical nets (Ø 25 cm, height 1.5 m). Before the start of the treatment the locust nymphs were starved for 24 hrs. Subsequently, they were allowed to feed for three consecutive weeks until harvest. Once per week, locust survival was determined. The first cohorts of the aphids were reared on white radish (*Raphanus sativus*) in transparent boxes (40 cm x 50 cm x 65 cm) which were stored in a climate room with conditions of 21°C, 14-hr light / 10-hr dark and 60% RH. We started with eight maternal lines, which were mixed in the last growth cohort to ensure sufficient genetic diversity before being transferred to the experimental plants. From the rearing only apterous adults with similar size were selected. Each replicate from each plant species also received five aphids and their total number was counted after 3 weeks.

In phase II, all pots with aboveground herbivory and soil feedback were completely randomized in the greenhouse. Plant biomass was determined as for the soil feedback experiment and relative herbivore effects were calculated within replicates as (shoot biomass with herbivores – shoot biomass without herbivores) / (shoot biomass without herbivores). We also determined locust survival and aphid population growth.

In order to test how the effects of aboveground herbivory and soil feedback were adding up, we compared the sum of the individual effects (predicted effect) with those of the treatments where plants were exposed to both soil feedback and aboveground herbivory (observed effect). Plant biomass in the control soil without herbivores was regarded as the biomass without any inhibitions. Then, the shoot and root biomass reduction for the soil feedback effect (difference between own and control soil) and the effect of herbivore reduction (difference between herbivory and no-herbivory treatment) were calculated for each plant species, added and compared with the treatment where plants were exposed to the combination of soil feedback and aboveground herbivory (Figure 2) (Haag *et al.*, 2004). Because plant biomass data are always positive they tend to follow a logarithmic distribution when biomass values approach zero, which would transform the linear additive effect towards a multiplicative effect (Slob, 1987). Moreover, in the treatment where plants were exposed to both soil feedback and aboveground herbivory, the impact of an aboveground herbivore also may have a negative effect on the belowground root biomass, and the soil community may have a negative effect on shoot biomass (Figure 1). Therefore, plotting the individual and combined effects on a logarithmic scale gives a more correct representation of the additive effect (Figure 2).

Statistical analyses
Effects of herbivores and soil pre-treatment on shoot biomass, root biomass and root/total biomass were analyzed using a full factorial ANOVA, including the effects of herbivore, soil, origin (native or exotic), species (nested in origin), and all their interactions. All variables were considered fixed, including species, because we used all species that were available in our experimental site; see the selection criteria as

explained above. Additionally, we performed an ANOVA in which we used species as units of replication. In that analysis, herbivory, soil, origin and all their interactions were used as factors in this analysis. Of primary interest is the soil x herbivory interaction, indicating whether enemy effects differ in their rank of effect size, as well as the origin x soil x herbivory interaction indicating whether the soil x herbivory interactions differ between native and range-expanding plant species. In order to test for a relationship between effects of aboveground herbivory and soil feedback a Spearman rank order correlation was performed with the species as replicate units. All statistical analyses were carried out in STATISTICA 9 (StatSoft, Inc., Tulsa, Oklahoma, USA). To improve normality and homogeneity of variances of residuals among groups defined by the statistical models, shoot biomass, root biomass and root biomass/total biomass were ln(x+1)-transformed prior to analysis.

Results

The effect of plant species and origin as separate factors had a significant effect on shoot biomass, root biomass and root biomass/total biomass (Table 2). Range expanding plants produced on average more shoot and root biomass than their native congeners (Figure 1a and 1b). Range-expanding plants overall had a higher proportion of root biomass than native plants (Figure 1c). Soil treatments 'own' versus 'control' had a significant effect on shoot and root biomass (Table 2). This soil effect could not be explained by differences in levels of available nitrogen or phosphorus between pots with own and control soil, nor between plant species (data not shown). Native plants had a significant reduction in their shoot and root biomass in own soil compared to control soil, whereas range expanders were less reduced (Figure 1a and 1b). Shoot herbivory reduced the shoot biomass of all plant species (Table 2, Figure 1a), and in some plant species shoot herbivory also resulted in less root biomass (Table 2, Figure 1b). In the analysis where species were treated as replicates the significances of the individual factors followed the same pattern (data not shown).

Native and range-expanding plant species had a significant species(origin) x soil interaction and a significant species(origin) x herbivory interaction in the shoots, as well as in the roots (Table 2). This indicates different responses among the plant species to the soil and herbivory treatments (see Figure S1 and Figure S2). The significant species(origin) x soil interaction in root/total biomass ratio pointed at different biomass allocation among plant species (Table 2). The significant origin x soil interaction in the root/total biomass ratio indicated that native and range-expanding plants differed in their root/shoot allocation in response to soil feedback (Table 2). This was due to a lower proportional investment in root biomass of native plants in own soil compared to the control soil (Figure 1c). In the analysis of root/total biomass ratio using species as replicates the origin effect remained, but the origin x soil interaction was non-significant ($F_{1,52} = 0.63$, $P = 0.432$). Overall, native plant species were reduced more (61% for shoots and 66% for roots) by the combined

effect of aboveground herbivory and soil feedback than range expanders (37% in shoots and 33% in roots) (Figure 1a and 1b). This explains the significant interaction effect of origin and herbivory on shoot biomass (Table 2). In the analysis using species as replicates the origin x herbivory interaction of shoots was not significant anymore ($F_{1,52} = 1.90$, $P = 0.174$).

There was no significant interaction between feedback from the soil community and aboveground herbivory neither for shoot nor for root biomass or root/total biomass ratio (Table 2). This indicates that soil feedback and shoot herbivory did not have synergistic or antagonistic interaction effects on the plant biomass, but that these belowground and aboveground effects add up linearly. In the analysis using plant species as replicates this interaction term also was insignificant. There was no significant three-way interaction between plant origin, soil feedback and aboveground herbivory for shoot and root biomass and root/total biomass ratio (Table 2). This shows that there is no allocation of biomass from the root to the shoot or vice versa as a compensation response to herbivore grazing, neither in the native plants nor in the range expanders. Moreover, there was no species(origin) x soil x herbivory interaction in shoot and root biomass and root/total biomass ratio. This indicates that there were no influences of aboveground herbivory on soil feedback or vice versa and that this is independent of the plant species considered (Table 2).

Table 2 ANOVA table of the effect of species nested in origin, origin, soil, herbivory and all their interactions on shoot biomass, root biomass and the root/total biomass ratio. All data were ln(x+1)-transformed prior to analysis. The effects of species(origin), origin, soil, interaction origin x soil, and the effects of species(origin), origin, herbivory, interaction origin x herbivory on total plant biomass were analyzed before in two separate analyses on two subsets of this dataset (Engelkes *et al.*, 2008).

|	shoot biomass			root biomass			root/total biomass		
	DF	F	p	DF	F	p	DF	F	p
species(origin)	13	27.2	<0.001	13	10.51	<0.001	13	19.09	<0.001
origin	1	173	<0.001	1	120.9	<0.001	1	56.74	<0.001
soil	1	47.5	<0.001	1	14.51	<0.001	1	2.98	0.055
herbivory	1	228	<0.001	1	29.35	<0.001	1	0.97	0.251
species(origin)*soil	13	3.22	<0.001	13	3.83	<0.001	13	3.10	<0.001
species(origin)*herbivory	13	11.5	<0.001	13	2.94	0.001	13	1.36	0.077
origin*soil	1	1.34	0.248	1	2.11	0.119	1	4.05	0.027
origin*herbivory	1	19.3	<0.001	1	0.06	0.882	1	2.44	0.080
soil*herbivory	1	1.35	0.247	1	0.00	0.890	1	0.15	0.759
origin*soil*herbivory	1	0.75	0.387	1	0.52	0.522	1	0.01	0.988
species(origin)*soil*herbivory	13	0.25	0.997	13	0.48	0.938	13	0.92	0.447
error	210			215			208		
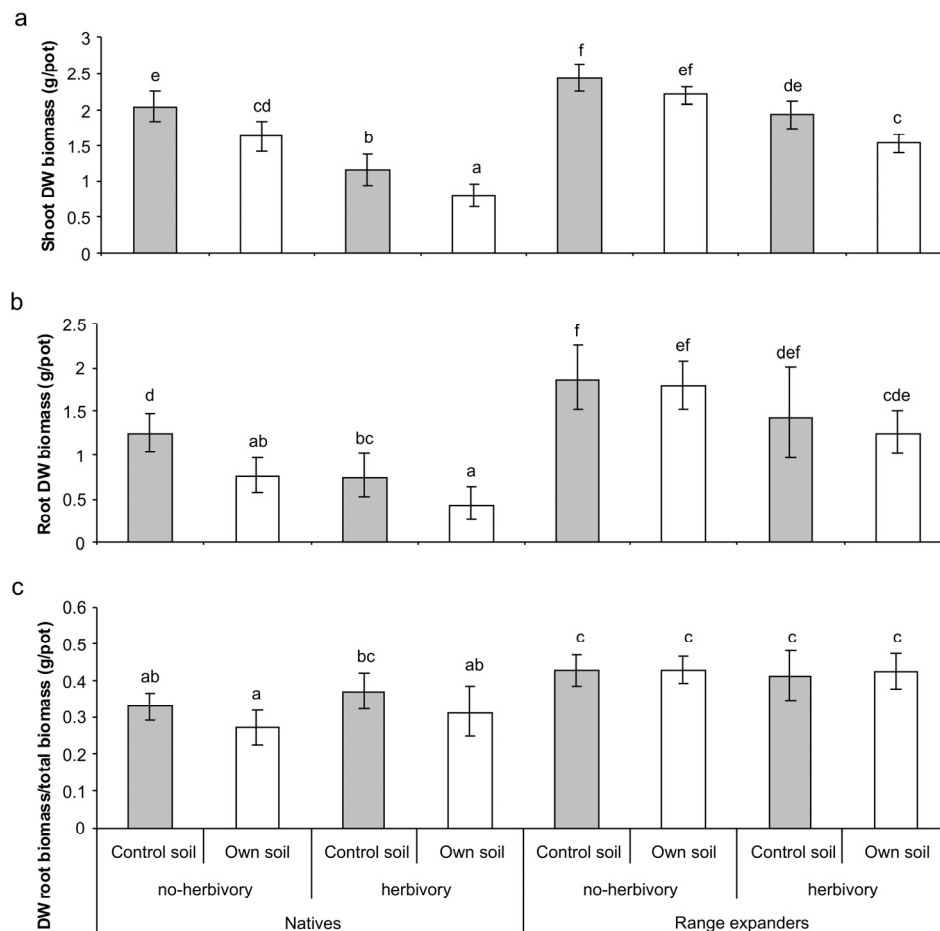
Figure 1 Upper panel a) Mean shoot dry weight biomass (g.pot^{-1} ± 1 s.e.) of native and range expanding plant species either or not exposed to herbivores and grown in pots containing own soil (white bars) or control soils (grey bars). Bars show back-transformed means of ln(x+1)-transformed data. Middle panel b) Mean root dry weight biomass (g.pot^{-1} ± 1 s.e.); same experimental design as in panel a. Lower panel c) Mean root dry weight divided by total dry weight (g.g^{-1}.pot^{-1} ± 1 s.e.) ; same experimental design as in panel a. In all panels means having the same letters are not significantly different at α = 0.05 according to a Tukey's HSD test.

There was no significant correlation between the relative soil feedback effects and the relative herbivore effects on the individual plant species (n = 6; R^2 = 0.4249, P = 0.257 for the range expanders and n = 9; R^2 = 0.1437, P = 0.333 for the native plants) (data not shown). Therefore, the effect size of aboveground herbivory could not predict the effect size of soil feedback and vice versa. However, the sums of the individual effects of aboveground herbivory only treatment and soil feedback only treatment corresponded well with the combined effect of aboveground and belowground herbivory, suggesting that under the present growth conditions combined effects can be estimated from determining shoot herbivory and soil feedback individually. This relation was even stronger in shoots (Figure 2a) than in the roots (Figure 2b). In Figure 2, the data of the range expanding species were closer to the basis of the graph than of the native plant species. This confirms that the range expanders are less harmed by aboveground herbivores and soil feedback than related natives.
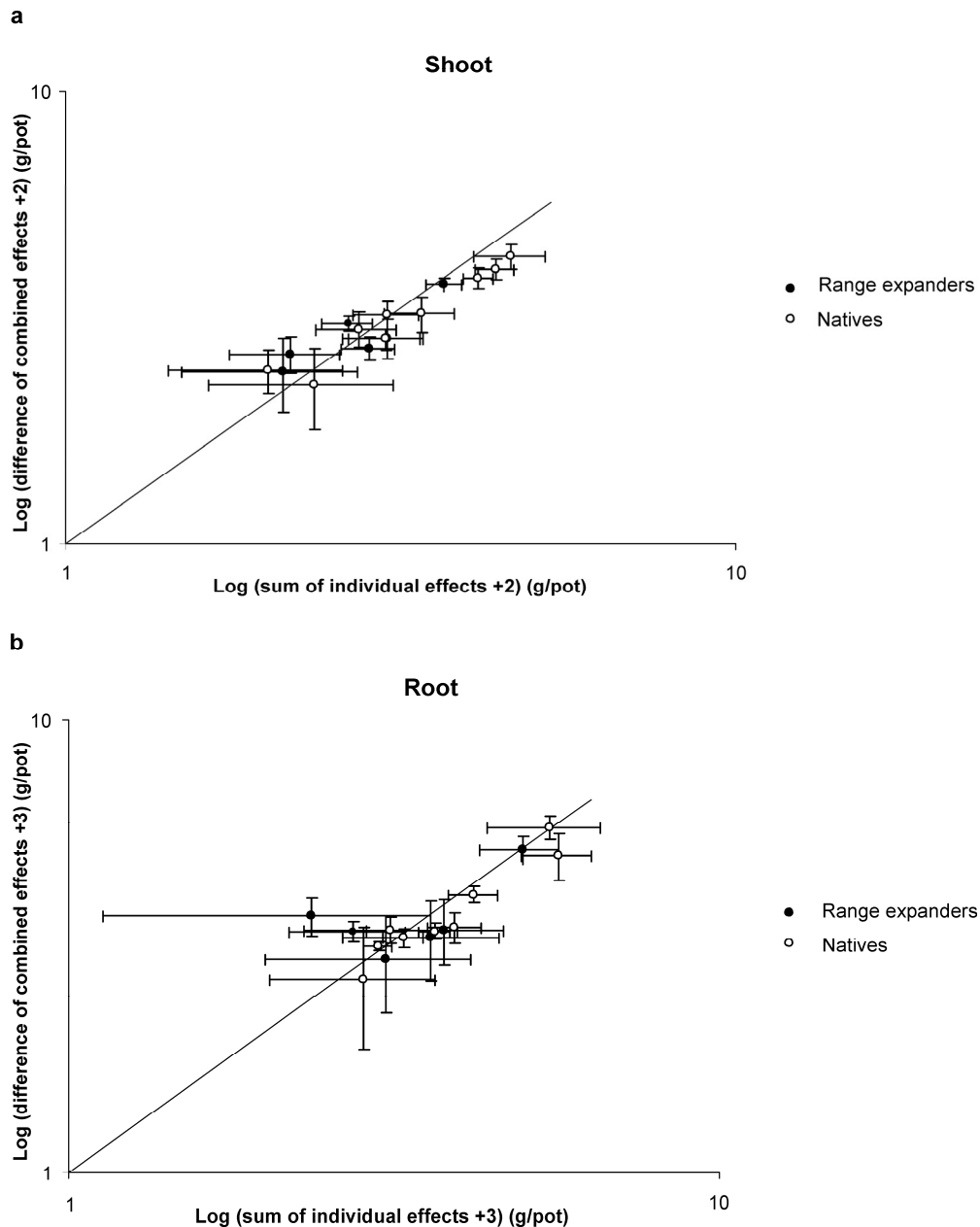
a

Shoot

b

Root

Figure 2 Sum of the individual effects of shoot herbivory and soil feedback plotted against the combined effects of shoots (upper panel a) and roots (lower panel b) of 9 native (open symbols) and 6 range expanding exotic plant species (closed symbols). In panel a, for the x-axis we used the average logarithm of the sum of individual effects of aboveground herbivores only and soil feedback only treatment + 2 to be able to plot positive values (originally based on plant biomass data expressed as g.pot^{-1} ± 1 s.e.; x-axis). These values were plotted against the logarithm of the difference between combined effects of aboveground herbivores and soil feedback and the control without above- and belowground herbivores + 2. The straight line represents a 1:1 relationship, which would be expected in the case of additive effects. From left to right symbols represent *Bidens tripartita, Bidens frondosa, Solidago gigantea, Angelica sylvestris, Angelica archangelica, Bidens cernua, Senecio inaequidens, Solidago virgaurea, Artemisia biennis* (overlaps with *S. virgaurea), Senecio viscosus, Senecio vulgaris, Centaurea stoebe, Centaurea jacea, Artemisia vulgaris* and *Centaurea cyanus*. In panel b, we added +3 in order to obtain positive values and from left to right symbols represent *Senecio inaequidens, Angelica archangelica, Angelica sylvestris, Bidens cernua, Bidens frondosa, Bidens tripartita, Solidago virgaurea, Solidago gigantea, Senecio vulgaris, Artemisia biennis, Centaurea cyanus, Senecio viscosus, Centaurea stoebe, Centaurea jacea* and *Artemisia vulgaris*.

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Discussion

In spite of the growing awareness that plant performance needs to be considered from a combined aboveground-belowground perspective (Bardgett & Wardle, 2010), little is known about how aboveground and belowground plant-enemy interaction effects work out at the level of plant individuals. We show that under our experimental conditions adding up the plant responses to the individual effects of aboveground herbivory and soil feedback closely approached the plant responses to combined exposure of aboveground herbivory plus soil feedback (Figure 2). Thus, our results suggest that combined effects of shoot herbivores and plant-soil feedback on plant biomass development are additive. Interestingly, range-expanding exotic plant species were influenced less by the aboveground and belowground enemies and still showed an additive response to combined exposure comparable to phylogenetically related native plant species. Our results also showed that enemy effects on plant biomass in one subsystem did not influence enemy effects in the other subsystem.

There was no correlation between the effect size of shoot herbivory and the strength of soil feedback. Thus, plant tolerance or resistance to polyphagous aboveground herbivores cannot be used as a predictor of plant sensitivity to soil feedback. These results further explain why in field studies aboveground and belowground enemy effects may not work out equally on all plant species present in a community (van Ruijven *et al.*, 2005), because plant species-specific differences in sensitivity to aboveground or belowground enemies can influence the outcome of plant-plant interactions. The plant species-specific sensitivities to aboveground and belowground enemies may result from, among others, the plant defense pathways involved (Bezemer & van Dam, 2005). Defense against aboveground feeders is mainly through the jasmonic acid pathway, whereas defense against belowground pathogens is regulated more via the salicylic acid pathway (Beckers & Spoel, 2006), which is a possible explanation why the strength of aboveground defense against insects does not need to correlate with the strength of plant-soil feedback.

In our study, we analyzed the interaction effects of shoot herbivory and soil feedback on single plants. In mixed plant communities plant species-specific aboveground and belowground enemy effects on plant individuals can influence the outcomes of plant-plant interactions, thus enforcing growth reduction initially due to plant enemies (Kardol *et al.*, 2007). Competition for abiotic factors, such as nutrients, light and water play an important role in plant-plant interactions and, therefore, have been used as key factors in explaining plant abundance (Grime, 1973; Grace & Tilman, 1990). However, these types of interactions need to be integrated with the roles that biotic interactions can have on plant competition, facilitation and coexistence. For example aboveground feeding by herbivores and selective effects of pollinators (Levin & Anderson, 1970; Carson & Root, 2000), as well as belowground effects from the soil community (Bonanomi *et al.*, 2005) all can be involved in influencing plant community composition, apart from resource availability. The

variety in responses among individual plant species aboveground or belowground will further enhance the number of possible outcomes of interactions within plant communities.

Our findings also help to further explain why promotion or slowing down succession depends on aboveground and belowground insect control, as well as on successional stage (Brown & Gange, 1989; Schädler *et al.*, 2004). Plants that are sensitive to shoot herbivory are not necessarily sensitive to growth reduction by soil biota and vice versa. Such plant sensitivities may vary both within and among succession stages. Our results are not in conflict with studies that have shown aboveground biota to influence belowground biota vice versa (e.g. West, 1995; Bardgett & Wardle, 2003), because those studies considered effects of aboveground and belowground second and higher trophic level organisms on each other, instead of considering the combined effects on plants, as we did in our study.

We conclude that aboveground and belowground control of plant biomass production by polyphagous shoot herbivores and plant-soil feedback can add up linearly when acting in concert. Interestingly, these additive effects were observed when considering both native and range-expanding exotic plant species, even though the range expanders were far less influenced by the aboveground and belowground plant enemies. However, the strength of negative soil feedback could not be predicted from the responses of the native and exotic range-expanding plants to polyphagous shoot-feeding insects, and vice versa. Population control of climate warming induced range-expanding plant species by aboveground and belowground enemies is still a new study area, but it can already be concluded that successful range expanders are less exposed to aboveground (Engelkes *et al.*, 2008) and belowground enemy effects (van Grunsven *et al.*, 2007, 2010; Engelkes *et al.*, 2008). Our results suggest that ultimately plant population control may originate from additive effects of aboveground and belowground enemies. Which of those enemies will provide strongest control may vary among plant species, due to species-specific differences in their sensitivities to shoot herbivory and soil feedback.

Acknowledgements

Supplementary results Individual plant shoot and root biomass responses to soil feedback and herbivory. See next two pages for figures S1 and S2.

Figure S1 Mean shoot dry weight biomass (g.pot-1 ± 1 s.e.) of range expanding and native plant species with or without shoot herbivores grown in pots with own soil (white bars) or control soils (grey bars). Bars show back-transformed means of ln(x+1)-transformed data. In all panels means having the same letters are not significantly different at α = 0.05 according to a Tukey's HSD test following ANOVA on species level with factors soil and herbivory.

Figure S2 Mean root dry weight biomass (g.pot-1 ± 1 s.e.) of range expanding and native plant species with or without herbivores grown in pots with own soil (white bars) or control soil (grey bars) treatments. Bars show back-transformed means of ln(x+1)-transformed data. In all panels means having the same letters are not significantly different at α = 0.05 according to a Tukey's HSD test following ANOVA on species level with factors soil and herbivory.
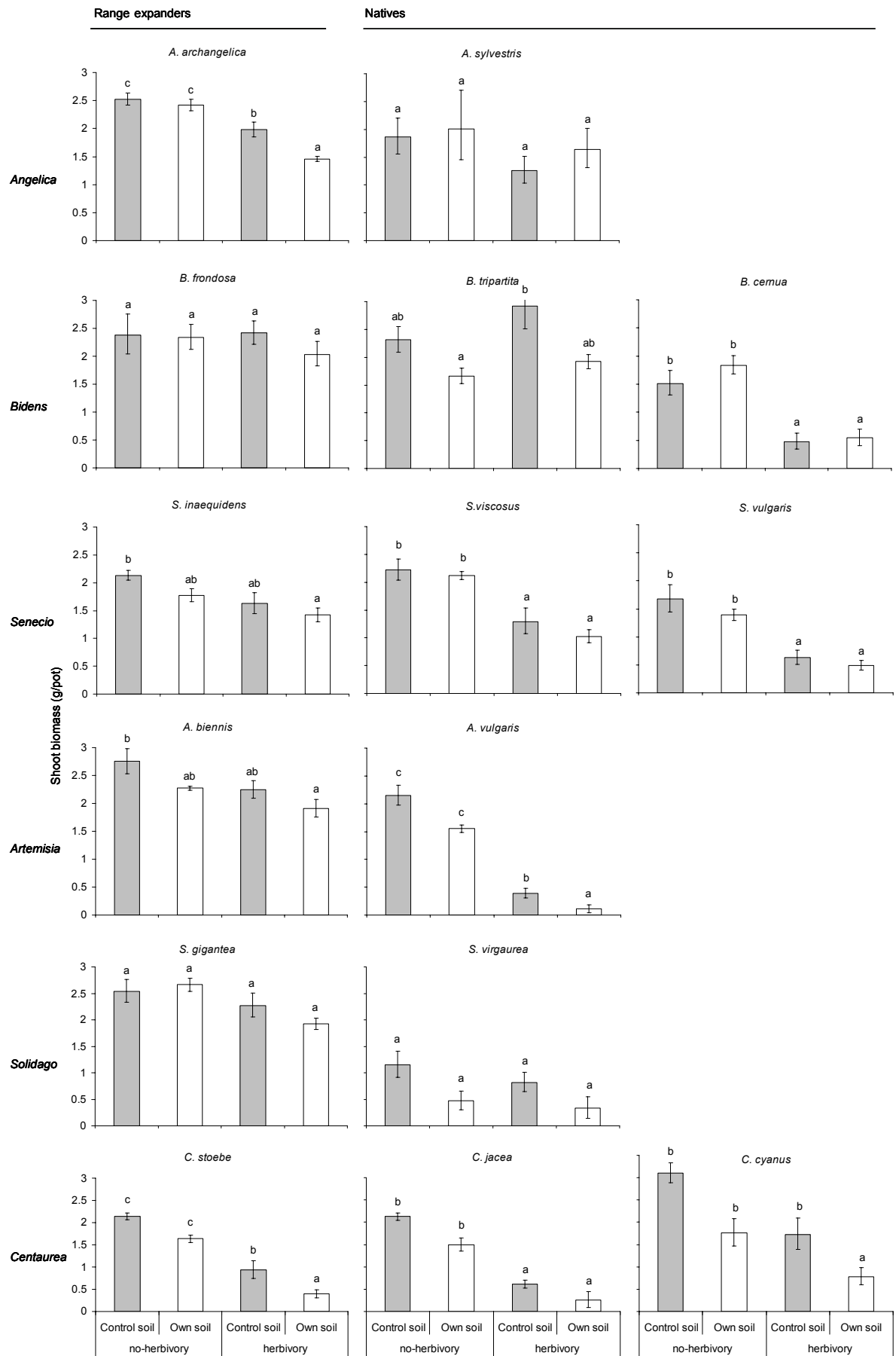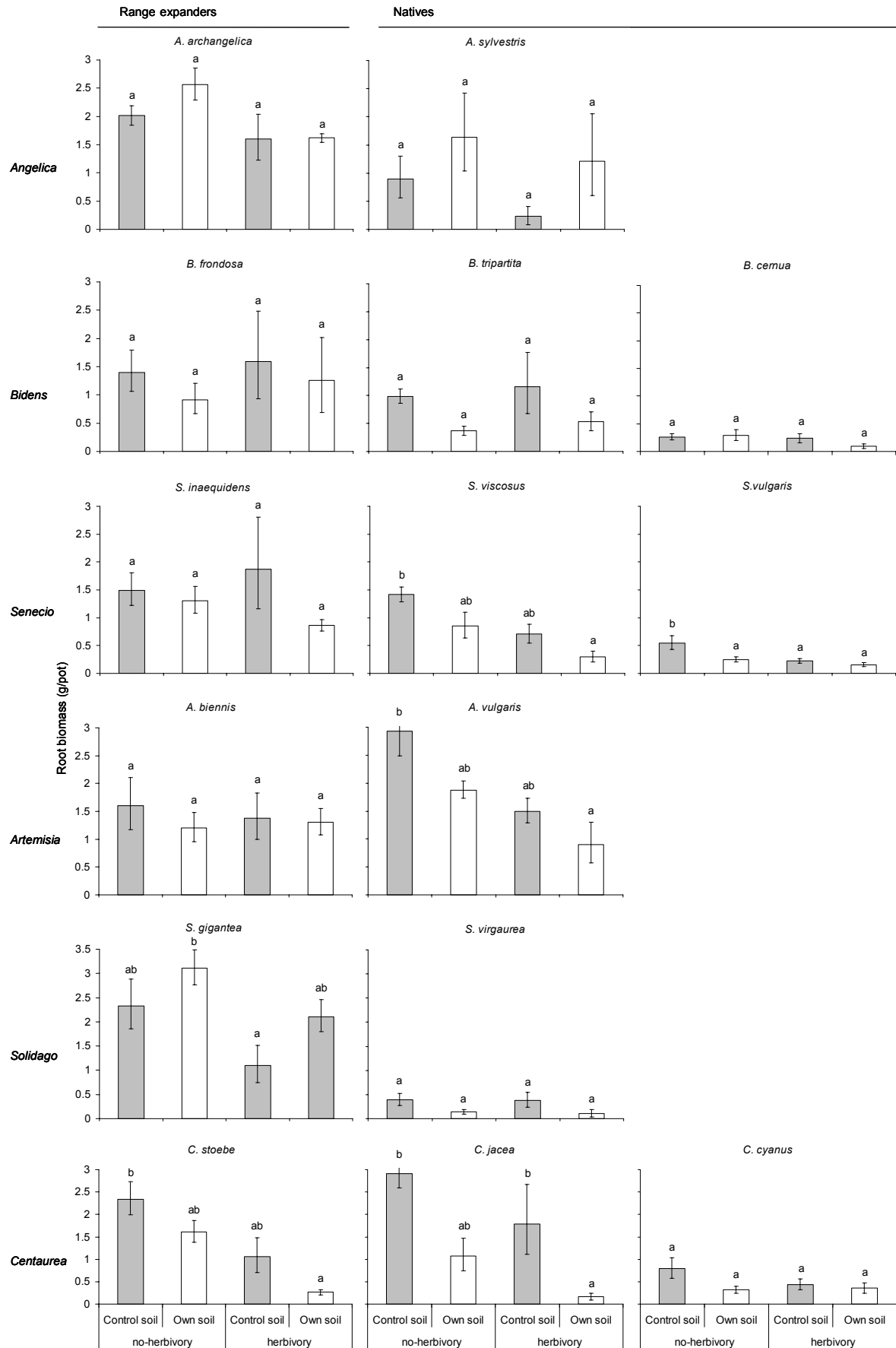
Chapter 4

EFFECTS OF NATIVE AND EXOTIC RANGE-EXPANDING PLANT SPECIES ON TAXONOMIC AND FUNCTIONAL COMPOSITION OF NEMATODES IN THE SOIL FOOD WEB

Elly Morriën, Henk Duyts & Wim H. van der Putten

Abstract

Due to climate warming, many plant species shift ranges towards higher latitudes. Plants can disperse faster than most soil biota, however, little is known about how range-expanding plants in the new range will establish interactions with the resident soil food web. In this paper we examine how the soil nematode community from the new range responds to range-expanding plant species compared to related natives. We focused on nematodes, because they are important components in various trophic levels of the soil food web, some feeding on plant roots, others on microbes or on invertebrates. We expected that range-expanding plant species have fewer root-feeding nematodes, as predicted by enemy release hypothesis. We therefore expected that range expanders affect the taxonomic and functional composition of the nematode community, but that these effects would diminish with increasing trophic position of nematodes in the soil food web. We exposed six range expanders (including three intercontinental exotics) and nine related native plant species to soil from the invaded range and show that range expanders on average had fewer root-feeding nematodes per unit root biomass than related natives. The range expanders showed resistance against rather than tolerance for root-feeding nematodes from the new range. On the other hand, the overall taxonomic and functional nematode community composition was influenced by plant species rather than by plant origin. The plant identity effects declined with trophic position of nematodes in the soil food web, as plant feeders were influenced more than other feeding guilds. We conclude that range-expanding plant species can have fewer root-feeding nematodes per unit root biomass than related natives, but that the taxonomic and functional nematode community composition is determined more by plant identity than by plant origin. Plant species identity effects decreased with trophic position of nematodes in the soil food web.

Introduction

Due to climate warming many plant species currently shift ranges to higher latitudes (Bakkenes *et al.*, 2002; Walther *et al.*, 2002; Parmesan & Yohe, 2003). When plant species shift range faster than organisms occupying higher trophic levels in the aboveground and belowground food web, range shifts may disrupt the original trophic interactions (van der Putten *et al.*, 2004; Menendez *et al.*, 2008; van Grunsven *et al.*, 2010). In their newly invaded range the range-expanding plant species can be considered exotic. Some of these range shifters originate from other continents, whereas others are native in lower latitude areas of the continent where range expansion takes place. All these plant species will establish new interactions with local aboveground and belowground biota. However, relatively little is known about the implications of plant range expansion for taxonomic and functional characteristics of food webs in the new range. Here, we study the response of nematodes from the invaded range to range expanding plant species in comparison with phylogenetically related plant species that are native in the invaded habitats.

Nematodes are important components of the soil food web, driving ecosystem processes (Ritz & Trudgill, 1999; Yeates, 1999; Griffiths *et al.*, 2000), influencing crop production (Yeates *et al.*, 2009) and plant community structure (e.g. van der Putten & Van der Stoel, 1998; Yeates, 1999; Verschoor *et al.*, 2002; De Deyn *et al.*, 2003). Besides root-feeding nematodes, there are nematodes feeding on protozoa, fungi, mites and other soil nematodes (Yeates *et al.*, 1993a). As nematodes are predominantly soil inhabitants, they are supposed to have less dispersal capacity and to respond less to climate warming by range expansion than many plant species (Berg *et al.*, 2010). Thus, when analyzing nematode community composition, an impression can be obtained of responses of the second, third, and higher trophic levels in the soil food web to range-expanding plant species. Previous studies on plants influencing nematode community composition have focused mostly on the relationship with plant species diversity and identity (Korthals *et al.*, 2001; Hedlund *et al.*, 2003; Wardle *et al.*, 2003; De Deyn *et al.*, 2004a; Viketoft *et al.*, 2005; Eisenhauer *et al.*, 2010) and less on effects of exotic plant species (van der Putten *et al.*, 2005). We investigated the taxonomic and functional community composition of nematodes in the root zone of range expanding versus related native plant species and compared effects of plant origin with plant species identity.

In some studies, important effects were found of plant identity on multiple trophic levels in the soil food web and ecosystem processes (Wardle *et al.*, 1999, 2003; Porazinska *et al.*, 2003). Primary consumers in the soil, such as microbes and root feeders can be more responsive to plant species composition than secondary (microbial feeders) and tertiary consumers (predators) (Wardle *et al.*, 2003; Viketoft *et al.*, 2009) Higher-level consumers on average are less diverse, less abundant, and under stronger anthropogenic pressure than primary producers (Duffy, 2002). This applies to a wide variety of above- and belowground biota (Cardinale *et al.*, 2006;

Scherber *et al.*, 2010), including nematodes at various trophic levels in the soil food web (Scherber *et al.*, 2010).

A reduced diversity (Brinkman *et al.*, 2005) or density (de Rooij-van der Goes, 1995; de la Peña *et al.*, 2008) of root-feeding nematodes can result in a lower feeding pressure on range-expanding plant species. This might explain why range-expanding plants receive less negative feedback effects from the soil community of invaded habitats than phylogenetically related native plant species (van Grunsven *et al.*, 2007; Engelkes *et al.*, 2008). Therefore, in the present study we also analyzed the relationship between the numbers of root-feeding nematodes per unit root biomass and the plant-soil feedback effects of range-expanding plants and plant species that are native in the invaded range. When range-expanding plants perform well while suppressing root-feeding nematodes, they can be considered resistant, but it is also possible that range-expanding plants perform well under high nematode densities (Trudgill, 1991). In that case they can be considered tolerant (Strauss & Agrawal, 1999). Besides these direct interactions of range expanding and native plant species with root-feeding nematodes, we also examined indirect interactions with nematodes at higher trophic levels in the soil food web. Exotic plants have been shown to alter soil biological and chemical characteristics in the rhizosphere, which can influence the structure of the microbial community (Kourtev *et al.*, 2003). This could have an indirect effect on bacterivorous- and fungivorous nematode community and their predators, through altered resource quality and quantity (Saj *et al.*, 2009).

We tested the hypothesis that range-expanding plant species have fewer root-feeding nematodes per unit root biomass and that the range-expanding plant species affect the taxonomic and functional composition of the nematode community different from phylogenetically related native plants. We compared plant origin effects with effects of plant species identity. We expected to find the strongest plant origin or plant identity effects on the plant feeders, and diminishing effects with increasing trophic position of nematodes in the soil food web.

Material and Methods

In order to determine the effects of plant origin and plant identity, we performed a growth experiment with range-expanding plant species and phylogenetically related natives using soil inocula from the invaded habitat. At the end of the growth experiment, the taxonomic and functional composition of the nematode community was analyzed in several ways. We compared the nematode abundance of the different feeding groups (root feeders, fungal feeders and bacterial feeders, omnivores and carnivores) and of feeding types within the root feeders: root associates (least specialized), ectoparasites (generally with wide host ranges), semi-endoparasites, migratory endoparasites (more specialized, but still often polyphagous) and sedentary endoparasites (containing both polyphagous species (e.g. *Meloidogyne* spp.) and more host-specific species (e.g. *Globodera* and *Heterodera*

spp.) (Trudgill, 1991; Yeates *et al.,* 1993a). Functional diversity of the nematode community was analyzed by diversity indices developed for nematode community studies (Ferris *et al.,* 2001).

Plant species, seeds and soil

We selected recently introduced range-expanding plant species and phylogenetically related natives using the National Standard List of the Dutch flora (FLORON 2003) and the updated version of this list (Tamis, 2005). In this database, the Netherlands is divided in grid cells of one square kilometer. Field surveys have been made to determine whether a grid cell is occupied by a certain plant species or not. We used a scale ranging from zero (no national grid cell occupied) to ten (all national grid cells occupied) (Tamis, 2005). We analyzed grid cell abundances between 1902-1950, between 1975-1988 and between 1988-2000 using four criteria: (1) range-expanding plants should have established in the Netherlands in the 20th century, and (2) they should have increased in national grid cell abundance in the last decades of the 20th century in order to have been able to respond positively to climate warming (Tamis, 2005), (3) they should have related native species in the same genus and (4) all native and range-expanding plant species occur in the same habitat (riparian areas in the Rhine Delta). These four criteria enabled us to produce a phylogenetically controlled comparison of native and recently invading range-expanding plant species (Agrawal *et al.,* 2005; Funk & Vitousek, 2007) sharing the same habitat. Of those plant species, we collected seeds from the field or, in a few cases, purchased seeds through a specialized seed supplier who collects seeds from local plant populations. Some species did not germinate, which left us with fifteen plant species in total, six range expanders and 9 natives.

Five out of the six range-expanding plant species belonged to the family *Asteraceae* (*Bidens frondosa, Senecio inaquidens, Artemisia biennis, Solidago gigantea* and *Centaurea stoebe)* and one to the *Apiaceae* (*Angelica archangelica*). Three range-expanding plant species originated from Eurasia: *A. biennis* (North-Asia)*, C. stoebe* (Central Europe) and *A. archangelica* (North East-Europe). The other three range expanders originated from other continents: *Bidens frondosa* (North-America)*, Senecio inaquidens* (South-Africa) and *Solidago gigantea* (North-America). We included nine related native plant species; for three genera we included two native plant species (*B. cernua, B. tripartita, S. viscosus, S. vulgaris, A. vulgaris, S. virgaurea, C. cyanus, C. jacea* and *A. sylvestris*).

Prior to germination, all seeds were surface sterilized using 100 ml diluted Loda Bleech (1% hypochlorid solution) for several minutes and then rinsing by demineralized water. Seeds were germinated on glass beads with demineralized water at day conditions of 14 hrs at 20°C and night conditions of 10 hrs at 10°C for species that flower in early season, or days of 16 hrs at 25°C and nights of 8 hrs at 15°C for species that flower in late season. Seedlings were placed at 4°C in light until all species had germinated and the experiment could start. After transplantation, dead seedlings were replaced until the third week of the experiment.

We collected soil from five random sites in the Millingerwaard (the Netherlands; 51°87′ N, 6°01′ E), a nature reserve in the Gelderse Poort region where all range expanding and related native plant species co-occur. The soil samples were homogenized, as we were not interested in spatial variation in the field, and inoculated into a sterilized sandy loam soil from Mossel, Planken Wambuis (52°06′ N, 5°75′ E). The soil sterilization was carried out by gamma radiation (25 kGray), which eliminated all soil biota (van der Putten *et al.*, 2007a).

Experimental setup
Phase I: soil conditioning. Seventy-five pots of 4 L were filled with a 5:1 mixture of sterilized soil and inoculum soil. We established five replicate pots of each plant species (six range expanders and nine natives, resulting in 75 pots). Each pot received four seedlings to promote soil conditioning and the experiment was carried out in a greenhouse under controlled conditions (60% RH, day: 21 ± 2°C; night 16 ± 2°C). Additional light was provided by metal halide lamps (225 µmol $^{-1}$ m^{-2} PAR) to ensure a minimum light intensity during the day. Plants were provided with demineralized water every second day to compensate for water uptake and evapotranspiration. Every week, initial soil moisture level was reset by weighing. In order to prevent plants from nutrient depletion, Hoagland solution was added at a rate of 25 ml of 0.5 strength week^{-1}, which is a dosage that does not prevent the establishment of arbuscular mycorrhizal fungi (van der Putten *et al.*, 2007a). After 8 weeks of growth, the plants were harvested and the conditioned soils were used for a second growth phase to test the feedback effect of their own soil community.

Phase II: soil feedback. The conditioned soil from every pot in phase I of the growth experiment was split into two halves. One half was placed in a 1.3-L pot to be called 'own' soil. The other half was used to create control soils. The control soil of every plant species contained soil conditioned by all other plant species, excluding plants from the same genus. Because all controls shared soil from five genera, we assumed initial soil nutrient conditions to be similar for all control pots. We established five replicates with own and five with control soils (resulting in 150 pots): each replicate was made from a separate replicate from the soil conditioning phase. We planted three seedlings per pot. Water, light and nutrient conditions were supplied as in phase I, except that 10 ml of 0.5 Hoagland solution was added on a weekly basis because the pots were smaller, there were fewer plants and there was less water loss from the soil surface compared to the 4 L pots. After week 10, when most nematode species should have had at least one reproduction cycle the pots were harvested. From half of the soil the roots were collected, air-dried at 70°C for 48 hrs and weighed. The other half of the soil and roots was used for nematode extraction, and thereafter the roots were air-dried and weighed. We calculated the soil feedback as ln[(total biomass in own soil)/(total biomass in control soil)], which provides symmetric positive and negative values (Petermann *et al.*, 2008; Brinkman *et al.*, 2010).

Nematode collection
Nematodes were extracted from those pots that contained own soil using Oostenbrink elutriators (Oostenbrink, 1960). The suspensions with nematodes were then led through one 75 μm sieve and three 45 μm sieves. The material, including nematodes collected from the 75 and 45 μm sieves was transferred to a double cotton filter (Hygia rapid, Hartmann AG, Heidenheim, Germany) on a sieve in a dish with a layer of tap water (Oostenbrink, 1960). The nematodes were allowed to migrate through the filter into the water for 24 hrs at room temperature, which resulted in relatively clean suspensions for nematode counting. Suspensions were stored at 4°C until they were fixated with hot paraffin. Root-inhabiting nematodes were collected by placing the roots in a mistifier. After nematode extraction for 48 hrs in the mistifier the roots were dried and weighted. The total numbers of nematodes in the root and soil samples were counted, identified to genus or family level using a reverse light microscope and categorized into feeding guilds according to Bongers (1988) and Yeates *et al.* (1993a). The root feeders were further subdivided into five feeding types: ectoparasites, semi-endoparasites, migratory endoparasites and sedentary endoparasites (Yeates *et al.*, 1993a). Epidermis or root hair feeders were classified as plant-associated nematodes (Yeates *et al.*, 1993b). Total numbers of nematodes were expressed as numbers per gram root biomass and numbers per pot.

Calculations and data analyses
The number of nematode taxa as used in Table 1 consists of mainly genera and some families and corresponds with the taxa mentioned in Figure 2, except for the five feeding types of the plant parasitic nematodes used. These were split into genera and one family (*Criconematidae*) (Table 1). Simpson's evenness was calculated to determine effects of plant identity on nematode taxonomic diversity (Magurran, 2004). Plant effects on the nematode community structure were assessed via the Structure index (SI) (Ferris *et al.*, 2001) and the Maturity index (MI) (Bongers, 1990) both indicating aspects of functional diversity. SI and MI are calculated as weighted abundances of nematodes in different feeding guilds, taking their position on the coloniser-persister (cp) scale into account. This scale ranges from 1 to 5; cp-1 nematodes are the enrichment opportunists with short generation times and a high reproduction rate, while cp-5 nematodes do not tolerate disturbance but have a long life span and low reproduction rate (Bongers & Bongers, 1998). The SI gives a value between 0 and 100 expressing the percentage of disturbance sensitive nematodes (mainly cp-3 values and onwards) per total nematodes (of all cp-values). The MI is calculated as the weighted mean of all cp-values from all taxa.

We analyzed the differences between origin and between plant species within origin using a nested ANOVA. All variables were considered fixed, including species, because we used all plant species that were available in our experimental site (Engelkes *et al.*, 2008). We performed Sign Tests to compare numbers of nematodes between natives and range expanders. These non-parametric Sign Tests are considered to be insensitive low-power tests, which provide strong evidence that

the main results from the overall analysis hold very generally across the plant species and nematode taxa within feeding guild (Siegel & Castellan, 1988). In order to test for a relationship between numbers of plant parasitic nematode per gram root and soil feedback a Spearman rank order correlation was performed with the species as replicate units. The same test was used to examine the relationship between omni-carnivore nematode numbers and the fungivorous, bacterivorous and root feeding nematodes with plant species as unit of replication. The plant root biomass against the number of plant-feeding nematodes was also tested by this approach. The effect of plant species identity on the nematode taxonomic (taxa, Simpson's evenness) and functional (SI, MI) diversity was tested by nested ANOVA where plant species were nested within origin. To compare nematode diversity among plant species Tukey HSD post-hoc tests were performed. All statistical analyses were carried out in STATISTICA 9 (StatSoft, Inc. 2009). To improve normality and homogeneity of variances of residuals among groups defined by the statistical models, numbers of root-feeders, fungivorous, bacterivorous and omni-carnivorous nematodes were ln(x+1)-transformed prior to analysis.

Results

There were no differences in numbers of root-feeding nematodes per pot between range expanding and native plants ($F_{1,51}$ = 1.19, P = 0.177) (Figure 1a). However, because range-expanding plants overall had more root biomass than native plants ($F_{1,51}$ = 57.16, P < 0.001; data not shown), they were exposed to fewer root-feeding nematodes per gram root biomass ($F_{1,51}$ = 15.93, P < 0.001) (Figure 1e). Within 7 out of 9 genera comparisons the range expanders had fewer root-feeding nematodes per gram root than the native plants (data not shown). This pattern also holds when considering the five feeding types (ectoparasites, semi-endoparasites, migratory endoparasites, sedentary endoparasites and plant-associated nematodes) separately. When considering the responses of the five feeding types to the nine plant genera separately, in 34 out of 45 (9 genera x 5 feeding types) comparisons there were fewer root feeders per gram root of the range expanders than of the natives (Z = 3.28, P = 0.001). The results of these 45 comparisons are not totally independent from each other, because all five feeding types occurred in each of the 9 comparisons. Nonetheless, the Sign test provides additional support to the ANOVA that roots of range-expanding plants were exposed to fewer root-feeding nematodes than roots of related natives.

There were more fungivorous nematodes per pot in range expanding than in native plants ($F_{1,51}$ = 4.48, P = 0.039) (Figure 1b). Due to more root biomass in range expanders than natives, range-expanding plants had fewer fungivorous nematodes per gram root than the native plant species ($F_{1,51}$ = 6.03, P = 0.018) (Figure 1f). When compared to the pattern observed for the root feeders, difference of numbers of fungivores per gram root biomass between range expanding and native plants was less pronounced. Within plant genus only in 5 out of 9 genera the range expanders

had fewer fungivorous nematodes per gram root than native plants. Considering the five genera of fungivorous nematodes separately the Sign Test gave no significant deviation from what could be expected by chance (data not shown).
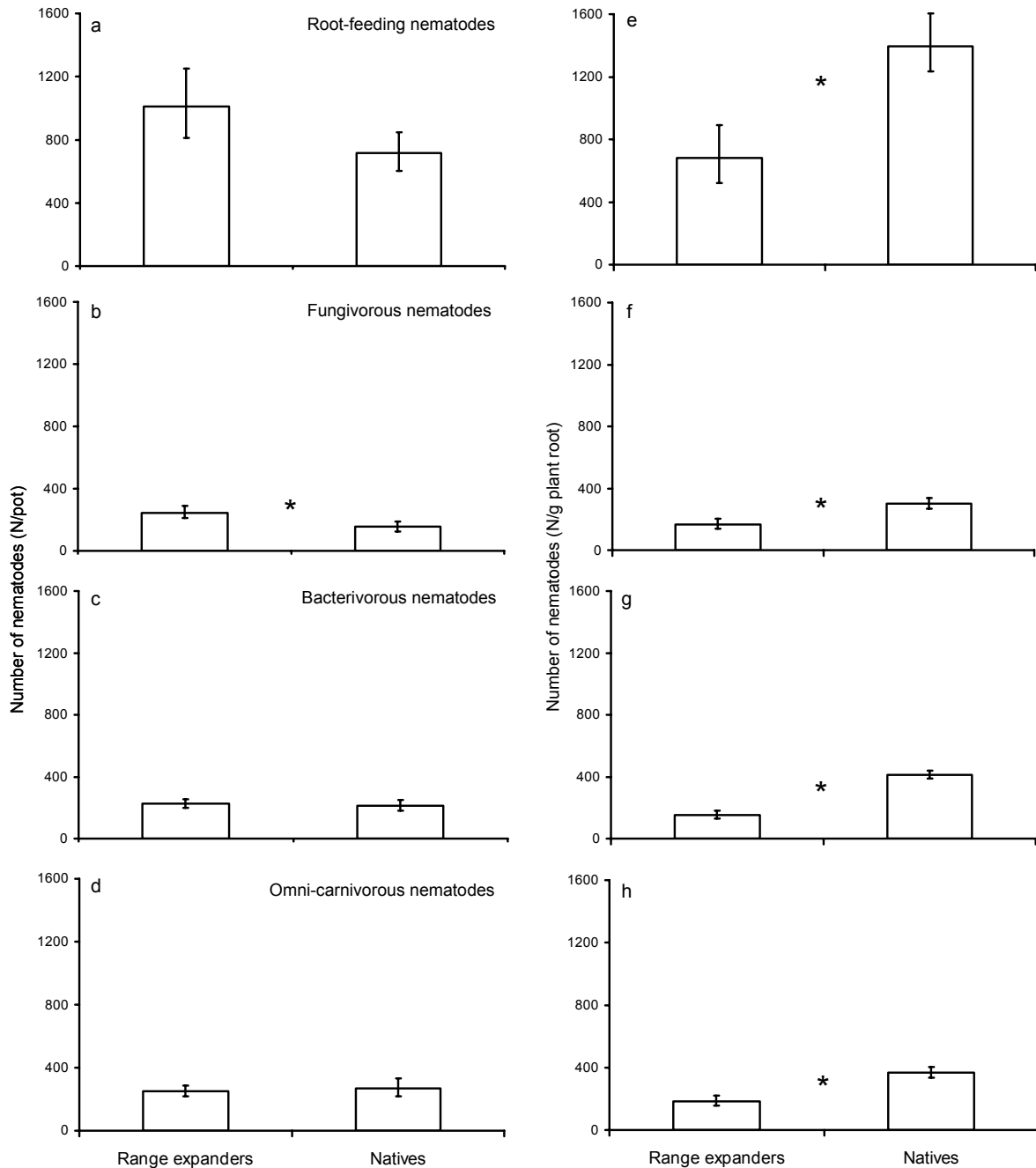
Figure 1 Panels a-d show mean nematode numbers per pot ± 1 s.e. in native and range expanding plants. Panels e-h show mean nematode numbers per gram root biomass ± 1 s.e. in native and range expanding plants. Panels a and e represent root-feeding nematodes, b and f fungivorous nematodes, c and g bacterivorous nematodes and d and h omni-carnivorous nematodes. Bars show back-transformed means of ln(x+1)-transformed data. ∗ indicates significant differences at $P < 0.05$.

Figure 2 Panels show mean nematode numbers per gram root biomass in the different plant species of native and range-expanding origin. Bars are divided into plant feeding types (panel a), or in family/genus level (panels b-d). $^+$ after species name on the x-axis indicates range-expanding plant species.

There were no differences in numbers of bacterivorous nematodes per pot between range expanding and native plants ($F_{1,51}$ = 0.08, P = 0.779) (Figure 1c). Since range expanders had more root biomass than natives, range expanders had fewer bacterivorous nematodes per gram root biomass than native plants ($F_{1,51}$ = 24.93, P < 0.001) (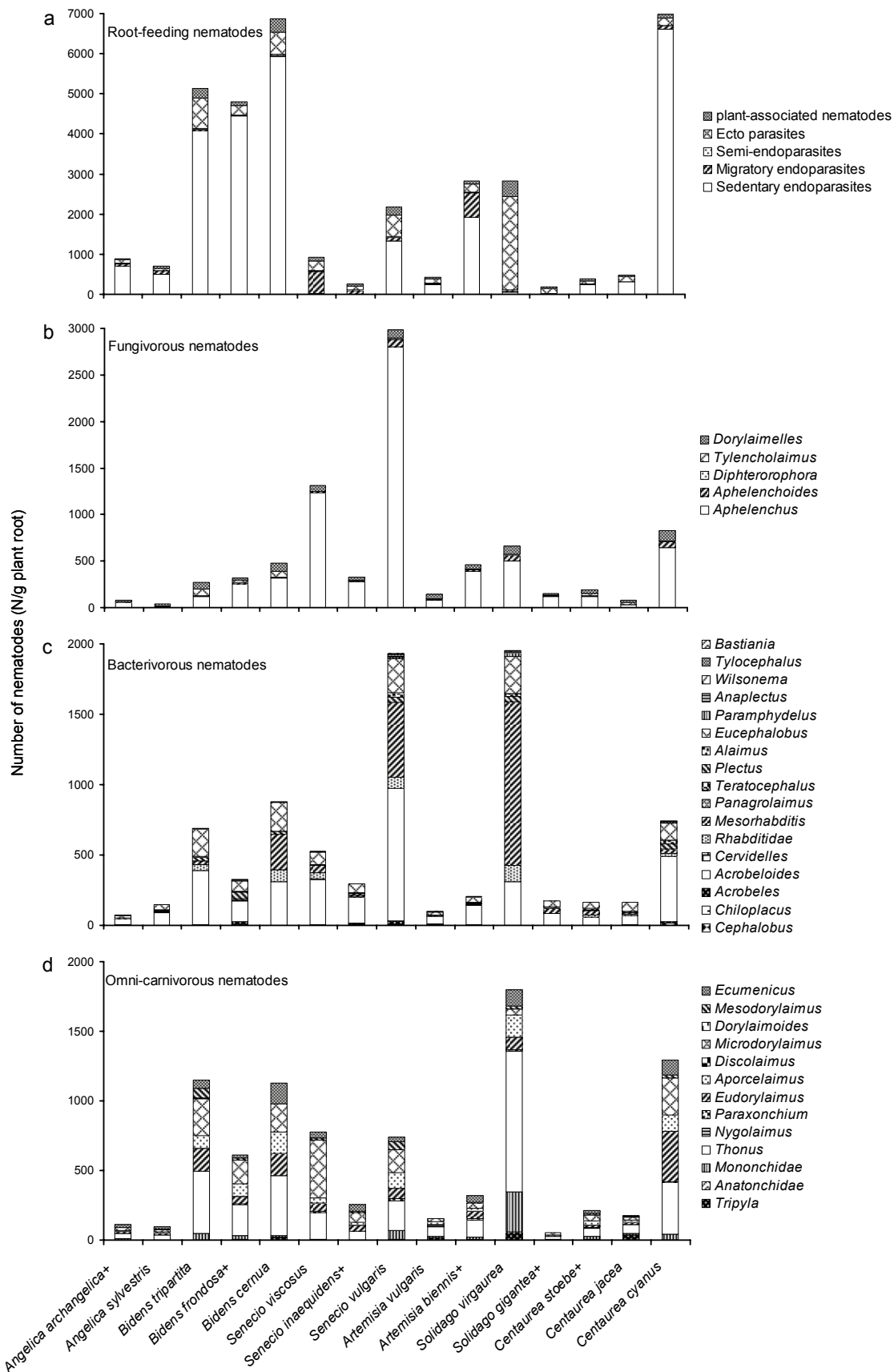Figure 1g). Within plant genus, in 7 out of 9 genera the range expanders had fewer bactivorous nematodes per gram root than native plant species. Within the sixteen taxa of bacterivorous nematodes in 68 out of 111 comparisons (9 genera x 16 bacterivorous nematode taxa minus 33 taxa that were not present in all genera) there were fewer bacterivores per unit root biomass in the range expanders than in the natives. A Sign Test made clear that this difference between native and range-expanding plant species is significantly greater than would be expected by chance (Z = 3.23, P = 0.001).

There were no differences in numbers of omni-carnivorous nematodes per pot between range expanding and native plants ($F_{1,51}$ = 0.24, P = 0.627) (Figure 1d). Since range expanders had more root biomass than natives, there were fewer omni-carnivorous nematodes per gram root biomass in the range expanders than in native plants ($F_{1,51}$ = 7.75, P = 0.008) (Figure 1h). When compared within plant genus, in 6 out of 9 nematode genera range expanders had fewer omni-carnivorous nematodes than native plants. Within the thirteen taxa of omni-carnivorous nematodes in 63 out of 96 comparisons (9 genera x 13 omni-carnivorous nematode taxa minus 21 taxa that were not represented in all genera) range expanders had fewer omni-carnivores than the natives. A Sign Test made clear that this difference between native and range-expanding plant species is significantly greater than would be expected by chance (Z = 3.16, P = 0.002). When we consider all feeding guilds of nematodes, we found the same trend in all guilds. Range expanders had fewer nematodes per gram root than native plants. This was not only caused by the higher root biomass of range expanders since the root biomass did not correlate with the root-feeding nematodes (n = 27, R^2 = -0.258, P = 0.195) (data not shown). Also the root biomass of the native plants did not correlate with the root-feeding nematodes (n = 39, R^2 = 0.193, P = 0.239) (data not shown).

Overall, root-feeding nematodes were the most numerous nematode guild (Figure 2). Sedentary endoparasitic nematodes (Figure 2a) represented the majority of the plant parasites in most plant species except for *S. viscosus*, *S. inaequidens* and both the *Solidago* species. Fungivorous nematodes were represented in lower numbers (Figure 2b), although they were relatively abundant in *S. viscosus* and *S. vulgaris*. Most fungivorous nematodes belonged to the genus *Aphelenchus*. The bacterivorous nematodes also occurred in smaller numbers than the plant parasites and were represented by a variety of different taxa (Figure 2c). *Eucephalobus*, *Mesorhabditis* and *Acrobeloides* were the dominant bacterivorous genera. The omni-carnivorous nematodes were also represented by a broader variety of taxa of which *Microdorylaimus*, *Eudorylaimus*, *Thonus* and the *Mononchidae* were the best represented taxa (Figure 2d). Overall, numbers of omni-carnivorous nematode per gram root biomass had a significant positive relationship with root feeders (n = 15, R^2
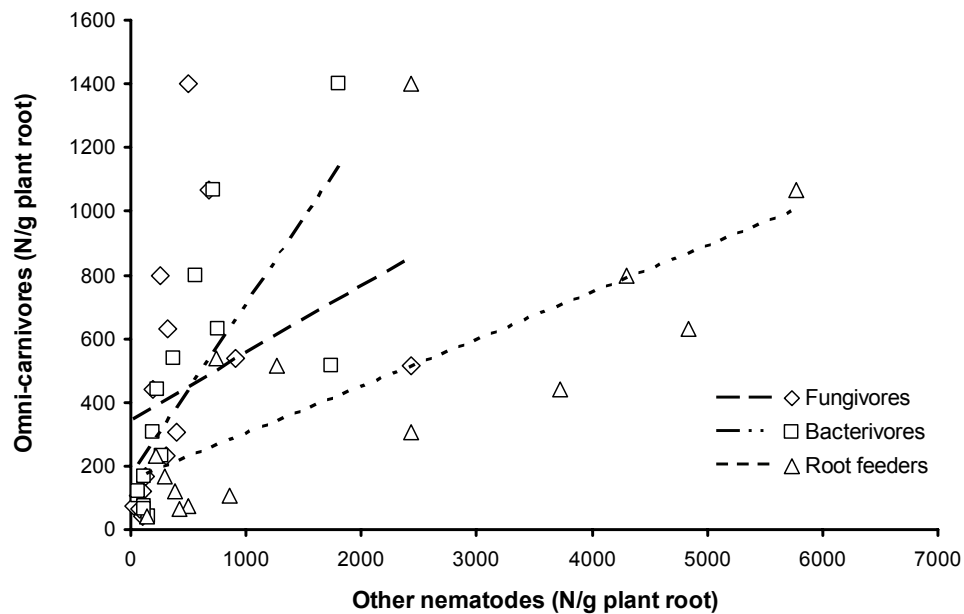
Figure 3 Relationship between the numbers of fungivores, bacterivores and root feeding nematodes per gram root biomass (x-axis) plotted against the number of omni-carnivore nematodes per gram root biomass (y-axis).

Table 1 Functional and taxonomic nematode diversity in pots with different plant species of native and range expanding origin (+ identifies range expanding plant species). Different letters denote differences at $P < 0.05$ level.

Species	Taxonomic diversity		Functional diversity	
	Number of taxa	**Simpson Evenness**	**Structure index**	**Maturity index**
Angelica archangelica +	26.4 ab	0.025 a	84.8 b	3.14 b
Angelica sylvestris	24.3 ab	0.031 ab	81.0 ab	2.94 ab
Bidens tripartita	23.0 ab	0.028 ab	86.4 b	3.23 b
Bidens frondosa +	20.6 a	0.024 a	83.6 b	3.18 b
Bidens cernua	22.3 ab	0.029 ab	82.9 b	2.95 ab
Senecio viscosus	26.3 ab	0.032 ab	71.6 ab	2.75 ab
Senecio inaequidens +	23.8 ab	0.037 b	71.8 ab	2.82 ab
Senecio vulgaris	24.0 ab	0.032 ab	49.5 a	2.25 a
Artemisia vulgaris	25.2 ab	0.033 ab	85.2 b	3.12 b
Artemisia biennis +	26.5 ab	0.027 ab	75.9 ab	2.96 ab
Solidago virgaurea	23.8 ab	0.034 ab	84.6 b	2.46 ab
Solidago gigantea +	22.3 ab	0.035 ab	64.6 ab	2.44 ab
Centaurea stoebe +	25.0 ab	0.034 ab	87.3 b	3.15 b
Centaurea jacea	28.0 b	0.032 ab	85.8 b	3.07 ab
Centaurea cyanus	22.8 ab	0.026 ab	78.0 ab	3.05 ab
Origin				
Native	24.4 a	0.031 a	77.9 a	2.87 a
Range expander	24.1 a	0.030 a	79.1 a	2.99 a

= 0.761, P = 0.001), fungivores (n = 15, R^2 = 0.793, P < 0.001) and bacterivores (n = 15, R^2 = 0.861, P < 0.001) (Figure 3). Plant species that had few root feeders per gram root also had few fungivores (although not significant) (n = 15, R^2 = 0.468, P = 0.079), bacterivores (n = 15, R^2 = 0.564, P = 0.028) and omni-carnivores (n = 15, R^2 = 0.761, P = 0.001) (Figure 3). When analyzed per pot, there was a tendency that plant species having most root-feeding nematode also had most fungivores (n = 15, R^2 = -0.157, P = 0.576), bacterivores (n = 15, R^2 = -0.45, P = 0.092) and omni-carnivores (n= 15, R^2 = 0.5, P = 0.058) (data not shown).

The taxonomic and functional diversity of nematode communities of range-expanding plants did not differ from natives (Table 1). However, there were some significant differences among plant species, mainly because *B. frondosa* had fewer nematode taxa than *C. cyanus* (Table 1). Simpson evenness of the nematode community of *B. frondosa* and *A. archangelica* was significantly lower than of *S. inaequidens* (Table 1). *S. vulgaris* had a significantly lower SI than *A. archangelica*, *B. tripartita*, *B. frondosa*, *B. cernua*, *A. vulgaris*, *S. virgaurea*, *C. stoebe* and *C. jacea* (Table 1). *S. vulgaris* also had a significantly lower MI than *A. archangelica*, *B. tripartita*, *B. frondosa*, *A. vulgaris* and *C. stoebe* (Table 1).

There was no relationship between soil feedback and numbers of root-feeding nematodes per gram root (n = 15, R^2 = -0.1571, P = 0.576) (Figure 4). However, the data reveal some interesting patterns. In Figure 4, three plant species (*A. sylvestris*, *A. archangelica* and *S. gigantea*) have a positive soil feedback and relative few plant root-feeding nematodes per gram root, suggesting that these plant species are resistant against nematodes. *Bidens cernua* showed tolerance, because it had a positive soil feedback while exposed to high numbers of root-feeding nematodes per gram root (Figure 4). *Bidens frondosa*, *B. tripartita* and *C. cyanus* had negative soil feedback coin-
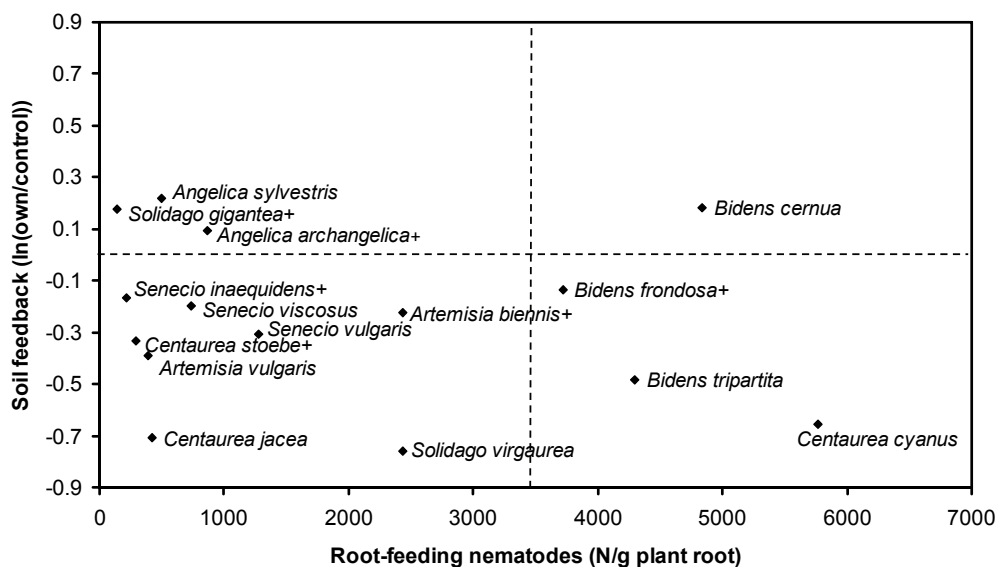

Figure 4 Mean numbers of root-feeding nematodes per gram root biomass (x-axis) plotted separately for each plant species against the soil feedback (y-axis). Soil-feedback was calculated for each replicate within plant species as ln[(total biomass in own soil)/(total biomass in control soil)]. Plant species are divided in four quadrants. + indicates range expanding plant species.

ciding with high amounts of root-feeding nematodes. These plants may be susceptible to root-feeding nematodes (Figure 4). Most other plant species in our experiment had a negative soil feedback while being exposed to relatively few root-feeding nematodes per gram root (Figure 4). In those cases root-feeding nematodes might have been contributing less to the negative soil feedback effects.

Discussion

Range-expanding plant species had fewer root-feeding nematodes per gram root biomass than phylogenetically related plant species that are native in the invaded habitat. The same pattern emerged for fungivorous nematodes, bacterivorous nematodes, and omni- carnivores (Figure 1e-h). However, the total amount of root-feeding nematodes per pot did not differ from pots with native plants, which is consistent with observations made by Porazinska *et al.* (2003). Since range expanders produced more root biomass per pot than their native counterparts, they had a lower exposure to nematodes per gram root biomass. During the 10 weeks of plant growth there should have been ample time for ectoparasitic root-feeding nematodes to have responded to the plant species by multiplication (de Rooij-van der Goes, 1995). On the other hand, the experimental period may have been relatively short for the sedentary endoparasites, some of which need up to 12 weeks to produce new cysts (Brinkman *et al.*, 2005). As differences between range expanding and native plants were quite consistent across the various types of root-feeding nematodes, our results suggest that the range-expanding plants promoted numbers of root-feeding nematodes less than related natives. Alternatively, it may be that nematode numbers in the rhizosphere of range-expanding plants are more controlled by natural enemies than in the rhizosphere of natives. This possibility is less likely, as it would require that the range expanders are able to develop novel interactions with higher trophic level organisms (Verhoeven *et al.*, 2009).

Although range expanders overall had fewer nematodes per gram root biomass than native plant species, plant identity appeared to be a stronger determinant of the numbers of root-feeding nematodes than plant origin. Within some genera, there was relatively little variation between species, such as was the case for *Bidens* spp., whereas in other genera, for example *Centaurea* spp., there was considerable variation among species (Figure 2). Therefore, both plant genus and species identity may determine the root-feeding nematode community composition more than plant origin. Effects of plant identity on the nematode community were also found by Yeates (1999), De Deyn *et al.* (2004a) and Viketoft *et al.* (2005), but none of these studies has considered exotic or range-expanding plant species.

A study on the foredune grass *Ammophila arenaria* in its non-native range showed that the grass had fewer specialized root-feeding nematode taxa than native grasses (van der Putten *et al.*, 2005). In our experiment sedentary endoparasites, which have relatively specialized interactions with their host plants, represented the majority of the root-feeding nematodes (Figure 2). However, most these sedentary

nematodes were *Meloidogyne* spp., which is a genus that contains relatively polyphagous species that can make good use of dicotyledon plants (De Deyn *et al.*, 2007). *Meloidogyne* spp. and other sedentary endoparasites were practically absent in *S. inaequidens* and *S. gigantea*, as well as in their related natives. In all other range-expanding plant species, there were high numbers of *Meloidogyne* spp. present, suggesting that these polyphagous root feeders perform relatively well on range-expanding plant species. On the other hand, more specialized sedentary endoparasites such as *Heterodera* were practically absent from all plant roots in our experiment, so that most feeding pressure will have been due to *Meloidogyne* spp.

Numbers of fungivorous nematodes were relatively high compared to other studies (Bardgett & Cook, 1998). These either were growing on fungi that have rapidly colonized the sterilized soil during the conditioning phase, or they may have been feeding on e.g. root hairs. Numbers of bacterivorous nematodes were similar to the fungivorous nematodes and are likely determined by the resource quality, rather than the resource quantity of the bacteria. Thus the nutrient content of the microbes can influence the microbivorous nematode community composition (Mikola & Setälä, 1998; Schmidt *et al.*, 2000). Omni-carnivorous nematodes were represented in similar numbers as the fungivores and the bacterivores and are determined by plant-feeding, fungivorous and bacterivorous nematodes as well as by the available microorganism community. Since many food sources are available for this group, the omni-carnivores seem to be the least responsive to the plant identity in the pot. Because of the duration of the experiment (10 weeks), we may have missed out on some taxa that have long reproduction cycles.

In order to obtain information on the effects of the range-expanding plant species on entire soil food webs, a more complete analysis of food web structure and functioning is required. Nevertheless, when we correlated the numbers of nematodes in the different feeding guilds to the numbers of omni-carnivorous nematodes bacterivorous nematodes turned out to explain more variation than fungivorous and root-feeding nematodes (Figure 3). In spite of our partial analysis of the soil food web, the data suggest that effects of plant origin, plant genus or plant species can trickle up the belowground food chain through nematode predator-prey interactions, being stronger through the bacterial (and slightly less through the fungal) based energy channel than through the plant feeder based energy channel. However, as bacterivorous nematodes responded less strong to plant differences than root-feeding nematodes plant origin and identity effects on soil food web interactions were found to diminish with increasing trophic position of the nematodes. These results also suggest that effects of range expanders on the decomposition channel in the soil food web are less pronounced than on the root feeder-based channel. Plant origin effects were weaker than for example effects of plant diversity determined in other studies (De Deyn *et al.*, 2004a; Viketoft *et al.*, 2005; Scherber *et al.*, 2010; Eisenhauer *et al.*, 2011).

There were no differences in taxonomic diversity and functional diversity between range expanders and native plant species (Table 1), however, there were

some differences due to plant species identities (Table 1). Most plant species, however, did not show differences in the number of nematode taxa. In an outdoor mesocosm study that was running for several years, plant species-specific effects on taxonomic diversity have been observed (De Deyn *et al.*, 2004a), so that such effects may require a longer duration of the experiment. There were some minor differences in Simpsons' evenness (Table 1) also implying that there were no major effects of plant species on the distribution of the nematode taxa and numbers per taxon. There were also some differences in functional diversity among plant species. Both SI and MI indicated that most nematodes in the community were somewhat disturbance sensitive, which would correspond to a grassland community with low disturbance frequency and a fungal-dominated decomposition channel (Ferris *et al.*, 2001).

A number of plant species in our experiment had negative soil feedback while having relatively few root-feeding nematodes (Figure 4). These plant species appear to be resistant to root-feeding nematodes, although these nematode numbers may also be low due to abundance control by competition or natural enemies (Piśkiewicz *et al.*, 2008). Most likely, root-feeding nematodes did not play a major role in these negative soil feedback effects, but selective elimination and inoculation studies are needed in order to further determine the relationship between nematode numbers and growth reduction (de Rooij-van der Goes, 1995). Some other plant species had high amounts of root-feeding nematodes in combination with negative plant-soil feedback (Figure 4). These plants appeared non-resistant and intolerant to root-feeding nematodes, but again selective elimination and inoculation studies are needed in order to determine the actual nematode effects on plant biomass production. Only one plant species had positive soil feedback in combination with high numbers of root-feeding nematodes (Figure 4), which may point at tolerance (Ashton & Lerdau, 2008). In any case, the range expanders did not reveal a specific strategy (tolerance or resistance), according to their scattered relationships between numbers of root-feeding nematodes and plant-soil feedback (Figure 4). These different strategies of range-expanding plant species to root-feeding nematodes may affect the development of virulence (Trudgill, 1991), which is the capacity of enemies to circumvent the resistance of the host species. Resistant plants tend to suppress the virulence of root-feeding nematodes while tolerant plants compensate biomass loss by compensatory growth (Trudgill, 1991). We have performed the plant-soil feedback experiment using an own-foreign comparison, which usually results in less outspoken feedback values than when using non-sterilized and sterilized soils as a comparison (Kulmatiski *et al.*, 2008; Brinkman *et al.*, 2010). Our observation that range-expanding plant species may differ in their interaction with root-feeding nematodes is not influenced by the way of testing plant-soil feedback effects; it only would have magnified the differences on the y-axis of Figure 4.

We conclude that range-expanding plant species are exposed to fewer root-feeding nematodes per unit dry root mass compared to their native congeners. However, the effects of range-expanding plant species on the nematode feeding guilds at higher trophic positions in the soil food web were less strong than for root

feeders. This suggests that effects of range-expanding plants diminish with trophic position of nematodes in the soil food web. Correlations between predator nematodes and their prey suggested that effects of range-expanding plant species may trickle up in the soil food web. This applies to both plant species originating from the same and other continents. The latter are being considered in most studies on invasive exotic plant species. Plant genus or plant species-specific effects on taxonomic and functional nematode community composition were stronger than plant origin effects.

Acknowledgements

Chapter 5

SOIL MICROBIAL COMMUNITIES OF EXOTIC RANGE-EXPANDING PLANT SPECIES DIFFER FROM THOSE OF PHYLOGENETICALLY RELATED NATIVE PLANTS

Elly Morriën & Wim H. van der Putten

Abstract

Due to global warming, many native and exotic plant species show range expansion from lower to higher latitudes. In the new range, associations of range expanding plants with soil microbes will establish that can have important ecological consequences. Here, we examine the rhizosphere microbial community composition of exotic range-expanding plant species in comparison with phylogenetically related native species. We tested the hypothesis that range-expanding plant species have a different soil community than related natives. In order to test this, soil was collected from the invaded habitat and six range-expanding and nine congeneric natives were planted individually in pots to condition soil microbial communities. After harvesting, individuals of the same species were planted in conditioned own and control soils. After ten weeks, we determined the rhizosphere community composition of bacteria, fungi, arbuscular mycorrhizal fungi (AMF) and fusaria. All groups of microbes were analyzed qualitatively using denaturating gradient gel electrophoresis. Ergosterol was determined as a quantitative measure of non-mycorrhizal fungal biomass and real-time PCR was applied to fusaria. Bacterial community composition was influenced by a combination of soil conditioning and plant origin, whereas fungal communities, AMF and fusaria were less pronounced in their responses to the experimental treatments. Range-expanding plants had less fungal hyphal biomass and lower amounts of fusaria in the rhizosphere than congenerics. We conclude that range-expanding plants have a different soil community composition than congenerics from the invaded habitat. The impacts of soil conditioning show that short-term plant growth may already lead to legacy effects in rhizosphere community composition.

Introduction

Due to climate change in the northern hemisphere, many native and exotic plant species are expanding their ranges northward (Walther *et al.*, 2002; Bakkenes *et al.*, 2002; Parmesan & Yohe, 2003; Pearson & Dawson, 2003; Tamis, 2005; Hickling *et al.*, 2006). During range expansion, interactions of plants and higher trophic level organisms can become disrupted because dispersal rates differ among species (van der Putten *et al.*, 2004; van Grunsven *et al.*, 2010; Thuiller *et al.*, 2007; Menendez *et al.*, 2008). A consequence of unequal dispersal rates is that range-shifting plants can become released from their native enemies (van Grunsven *et al.*, 2007, 2010; Engelkes *et al.*, 2008). As soil biota are slow dispersers (Berg *et al.*, 2010), soil communities in the new range may not necessarily contain pathogens, decomposers and symbiotic mutualists from the native range. Compared to native plants, enemy release may cause a competitive advantage for exotic plants species in the invaded habitat (Walker *et al.*, 2003; van der Putten *et al.*, 2007b; Inderjit & van der Putten, 2010), whereas non-adapted mutualists and decomposers may cause a disadvantage for plants that have moved away from their home fields (Richardson *et al.* 2000; Chapman & Koch, 2007; Ayres *et al.*, 2009; Pregitzer *et al.*, 2010). The question that we will address in the present study is how the rhizosphere community of exotic range-expanding plant species in the new range compares to congeneric plant species native in the invaded habitat.

In a previous study, soil communities from the invaded range were less pathogenic to range-expanding plant species than to phylogenetically related species that were native in the invaded habitat (Engelkes *et al.*, 2008). The neutral feedback between soil microbiota from the invaded range and exotic plants (Klironomos, 2002; Reinhart *et al.*, 2003; Callaway *et al.*, 2004; Bennett *et al.*, 2006; Pringle *et al.*, 2009) may be due to a shifted balance towards relative absence of pathogens and accumulation of mutualistic fungi in the roots and rhizosphere of exotic plants. Such soil community effects are determined by measuring feedback effects after a phase of conditioning by the same or other plant species (Bever *et al.*, 1997). Negative feedback between plants and soil communities is a widespread phenomenon in native vegetation and this so-called plant-soil feedback contributes to plant diversity and coexistence (Bever *et al.*, 1997; Klironomos, 2002; Bever, 2003; Petermann *et al.*, 2008).

Exotic plants that have been moved from one to another continent can be released from negative soil feedback (Klironomos, 2002). This release has been proposed to promote plant invasiveness (Reinhart & Callaway, 2006). Studies on aboveground pathogens of exotic plants have shown that recently introduced exotic species have fewer pathogens than exotic plant species with a longer residence time (Mitchell *et al.*, 2010). When time since invasion increases, soil-feedback effects can become increasingly negative (Diez *et al.*, 2010). Besides, soil-feedback effects can remain to be effective in the long term (Kulmatiski & Beard, 2011). Based on these

studies, we would expect that the rhizosphere of currently range-expanding exotic plants may also contain fewer soil pathogens than phylogenetically related natives.

Root exudates and rhizo-deposits form the substrates for rhizosphere bacteria and fungi, and it has been recognized that the composition of these substrates can differ among plant species (Nelson, 1990). Differences in root-derived substrates are believed to explain the plant species-specific rhizosphere bacterial communities that have been observed for different plant species under otherwise similar conditions (Marschner *et al.*, 2001; Kowalchuk *et al.*, 2002; Carney & Matson, 2005; Hartmann *et al.*, 2009). Similar growth conditions are important when comparing rhizosphere communities (Berg & Smalla, 2009; Eisenhauer *et al.*, 2011) since root exudate composition is also affected by soil conditions such as pH, nutrient limitation, soil moisture and exposure to pathogens (Yang & Crowley, 2000).

Both bacteria and fungi respond to plant community composition and processes in the rhizosphere (Gomes *et al.*, 2003; de Boer *et al.*, 2006). We studied differences in soil communities of general bacteria and fungi to obtain a first overview of microbial shifts due to soil conditioning and plant origin. In addition, we examined the effects of exotic range-expanders on the soil community by studying specific effects on two taxonomic groups of microbes, one including symbiotic mutualists (arbuscular mycorrhizal fungi; AMF) and the other including the genus *Fusarium*, which contains a number of economically important plant pathogenic species that can cause substantial damage to crop plants. They are a large genus of fungi widely distributed including relatively abundant members of the rhizosphere microbial community (Booth, 1971). In addition and in contrast to the host-specific nature of many pathogenic microbes, we included AMF because many AMF taxa tend to infect a broad range of host plants (Eom *et al.*, 2000).

In order to analyze the microbial community structure across a wider range of plant species we compared rhizosphere microbial community structure of six exotic range-expanding plant species to nine phylogenetically related natives. We hypothesized that soil microbial composition of range-expanding plant species differs from their native congeners due to relative absence of pathogens and accumulation of AMF in the roots and rhizosphere of range-expanding plants. All groups were analyzed qualitatively using denaturating gradient gel electrophoresis (DGGE). We determined ergosterol as a quantitative measure of non-mycorrhizal fungal biomass and applied real-time PCR to fusaria. In line with our hypothesis, we expected to find lower amounts of ergosterol and fusaria in the rhizospheres of range-expanding plant species than of natives.

Material and Methods

Plant species selection
We used the National Standard List of the Dutch flora (FLORON, 2003) and the updated version of this list (Tamis, 2005) to select recently introduced exotic range-expanding plant species and phylogenetically related natives (Agrawal *et al.*, 2005;

Funk & Vitousek, 2007) all co-occurring in a riverine habitat in eastern Netherlands (51°87′ N, 6°01′ E). Three exotic plant species originated from Eurasia: *Artemisia biennis* (North Asia), *Centaurea stoebe* (Central Europe) and *Angelica archangelica* (North East Europe). The other three range expanding exotics originated from other continents: *Bidens frondosa* (North-America), *Senecio inaquidens* (South-Africa) and *Solidago gigantea* (North-America). We compared the exotic plants with native plant species (*Bidens cernua*, *Bidens tripartita*, *Senecio viscosus*, *Senecio vulgaris*, *Artemisia vulgaris*, *Solidago virgaurea*, *Centaurea cyanus*, *Centaurea jacea* and *Angelica sylvestris*). In three genera, we included two species in order to test the sensitivity of our analysis to species-specific effects on rhizosphere community composition.

Experimental setup

In order to determine if short-term plant species legacy effects were already of influence on rhizosphere microbial community structure, we first conditioned the soil (phase I) and did the actual measurements in the soil-feedback phase II. This is a common approach for plant-soil feedback experiments (Bever *et al.*, 1997).

 Phase I: soil conditioning. Seventy-five pots of 4 L were filled with a 5:1 mixture of sterilized soil and living inoculum soil. We established five replicate pots of each plant species (six exotics and nine natives, resulting in 75 pots). Each pot received four seedlings to promote soil conditioning and the experiment was carried out in a greenhouse. After 8 weeks of growth, the plants were harvested and the conditioned soils were used for a second growth phase to test the feedback effect of soil community conditioning.

 Phase II: soil feedback. The conditioned soil from every pot in phase I of the growth experiment was split into two halves. One half was placed in a 1.3 L pot to be called 'own' soil. The other half was used to create control soils. The control soil of every plant species contained soil conditioned by all other plant species, excluding plants from the same genus. Because all controls shared soil from five genera, we assumed initial soil nutrient conditions to be similar for all control pots. We established five replicates with own and five with control soils (resulting in 150 pots). After week 10, the pots were harvested, and three-quarters of the roots were collected to estimate the total amount of root dry biomass. Aboveground biomass and roots were air-dried at 70°C for 48 hrs and weighed. The other quarter of the soil and roots was used to collect root and rhizosphere samples according to Kowalchuk *et al.* (2002). Briefly, we collected roots from the soil and removed the bulk soil by gently shaking roots using tweezers. Rhizosphere soil was collected by brushing off the soil particles from the roots. The remaining roots with tightly attached soil particles were cut with scissors and stored in an eppendorf tube at -80°C. All equipment used was sterilized in between sampling using 98% ethanol.

DNA isolation and PCR-DGGE analyses

We extracted genomic DNA from approximately 0.25 g (wet weight) of the root and attached soil particles using the PowerSoil™ DNA isolation kit (MoBio Laboratories,

Carlsbad, CA, USA). Prior to isolation, root samples were ground in a mortar under liquid nitrogen. The composition of the fungal, AMF and fusaria community was studied using the fungal 18S rRNA gene-specific primers, for the bacterial composition we used the 16S rRNA gene-specific primers (Table 1). Details regarding primers, thermocycling regimes and electrophoresis conditions are listed in Table 1, and all PCR was performed with a PTC-200 thermal cycler (MJ-Research, Waltham, MA. Each amplification reaction mixture (25 μl) consisted of 0.5 μl (30 pM) of each primer, 2.5 μl 10 × PCR-buffer, 2.5 μl (2 mM) of the dNTPs mix, 0.40 μl (0.056 U) Expand High Fidelity DNA polymerase (Roche, Mannheim, Germany), 1 μl template DNA (between 15-95 ng depending on the plant species), and 17.60 μl MilliQ (Millipore BV, Etten-Leur, the Netherlands).

All thermocycling programs were preceded by an initial denaturation step (95°C for 5 min) and followed by a final elongation step phase (72°C for 10 min). Each PCR cycle consisted of a denaturation step at 95°C for 1 min, an annealing step at the specified temperature (Table 1) for 1 min and an elongation step at 72°C for 1 min. Touchdown protocols started with the highest annealing temperature, which was subsequently lowered by 2°C after every 2 cycles until the target annealing temperature reached. The amplicons of fusaria were diluted (1:1000) and reamplified in a second PCR round (Table 1). PCR products were examined by standard 1.5% (w/v) agarose 0.5 × TBE gel electrophoresis with ethidium bromide staining to confirm product integrity and estimate yield.

The composition of the fungal and bacterial communities was characterized using denaturating gradient gel electroforesis (DGGE), using the method of Muyzer *et al.* (1993) as modified by Kowalchuk *et al.* (2002) except that the linear gradients were as indicated in Table 1. To ensure well-polymerised slots, a 10 ml top gel containing no denaturant was added before polymerization was complete. Denaturating gels were prepared with the gradient former Bio-Rad model 230 (Bio-Rad Laboratories, Veenendaal, the Netherlands) at a speed of 5 ml/min. A GC-rich sequence (indicated as -gc) was attached to one of the primers in the set to prevent total melting of PCR products during separation in the denaturating gradient gel.

All DGGE analyses were run using a D-Gene system (Bio-Rad Laboratories, Hercules, CA, USA). Total PCR products (25 μl) were applied to the gel, and DGGE was performed in 1 × TAE buffer at 60°C. Electrophoresis was for 10 minutes at 200 V, after which the voltage was lowered to 70 V for an additional 16 hrs. For comparison of banding patterns, a reference marker was added in triplicate to each gel. Gels were stained in MilliQ water containing 0.5 mg/l ethidium bromide and destained in MilliQ water prior to UV transillumination. Gel images were digitally captured using the ImaGo system (B&L Maarsen, the Netherlands). To facilitate comparative statistical analyses, all gels of the same community were combined into a composite image using Corel PHOTO-PAINT 12 prior to further analysis (Corel Corporation, 2003). Banding patterns were normalized with respect to standards of known composition as well as samples loaded across multiple gels using the Image Master 1D program (Amersham Biosciences, the Netherlands).

Real-time PCR

Real-time PCR was performed using the ABsolute QPCR SYBR green mix (AbGene, Epsom, UK) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) to quantify fusaria 18S rRNA gene copies. The mixes were made using a CAS-1200 pipetting robot (Corbett Research, Sydney, Australia). Quantification of *Fusarium* gene copies in the rhizosphere was carried out as described in Table 1 except that the forward primer lacked a GC-clamp. Standards were made from full length PCR-amplified 18S rRNA genes from a pure fusaria colony. To make the fusaria standard, DNA was extracted using the PowerSoil™ DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), PCR-amplified and cloned. One resulting clone that contained a proper insert of fusaria origin was randomly chosen and used in a colony PCR-procedure using plasmid enclosed primer. The resulting PCR-product was purified and quantified on a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the number of gene copies/µl was calculated using the molecular weight of one genome as calculated from sequences deposited in GenBank. DNA extracts from the rhizosphere samples of the plants species used in our experiment were all diluted to 5 ng/µl of which 2.5 µl was used for real-time PCR. Using 10-fold increments, the standard concentrations were adjusted from 10^6 to 10^1 SSU rRNA gene copies/µl. All samples and standards were assessed in two different runs to confirm the reproducibility of the C_T values. The results of the different runs were read out at a threshold of 0.52 where the efficiencies of the different runs were most comparable.

Ergosterol measurements

We used the alkaline ergosterol extraction described by (Bååth, 2001) with minor modifications as described in (de Ridder-Duine *et al.*, 2006).

Table 1 Primers, PCR and DGGE conditions used in this experiment.

Community	Primers	PCR-protocol	DGGE gradients	Reference
Bacteria	968-gc/1378	Touchdown 65 to 55°C; 35 cycles	45 – 65% denaturant	Heuer *et al.*, 1997
Fungi	FR1-gc/FF390	Touchdown 55 to 47°C; 37 cycles	40 – 55% denaturant	Vainio & Hantula, 2000
Mycorrhizae	LR1(-f)/FLR2(-R) Followed by FLR3/FLR4	Touchdown 1st 58°C; 35 cycles 2nd 58°C; 35 cycles	20 – 60% denaturant	Gollote *et al.*, 2004
Fusaria	EF-1/EF-2 Followed by Alfie1-gc/Alfie2	Touchdown 1st 50°C; 29 cycles 2nd 67°C; 34 cycles	40 – 60% denaturant	Yergeau *et al.*, 2005

[1] PCR-protocols are given as: annealing temperature; number of cycles. The remaining of the procedure is given in the text.
[2] 100% denaturant is defined as 40% (v/v) formamide and 7 M urea.

Statistical analyses

We analyzed the DGGE gels using binary data, presence or absence of bands, generated by the Image Master 1D program (Amersham Biosciences, the Netherlands). The resulting binary matrices were exported and used in statistical analyses as 'species' presence-absence data. Multivariate tests of significance of the effects of soil treatment and plant origin in DGGE patterns were carried out using a Principal Coordinate Analysis (PCoA) using a Jaccard index followed by a distance-based Redundancy Discriminant Analysis (db-RDA) for the analysis of species, genus, soil treatment, plant origin and interaction effects (Legendre & Anderson, 1999). We implemented the experimental design as split-plots (soil treatments) within a whole-plot (plant species) using 499 random permutations in the Monte Carlo permutation test in CANOCO 4.5 (ter Braak & Šmilauer, 2002). When we were interested in the effects on the species level (species, genus and plant origin), we permuted whole-plots, but not split-plots. When we were interested in the effect of the soil treatment, we permuted the split-plots, but not whole-plots. The interaction between plant origin and soil treatment was analyzed regarding species as fixed effects in a Welch-test where the same split-plots and whole-plot were both permuted at random (Welch, 1947). Data were visualized as RDA ordination scatterplots. When plotting samples we made sure to derive the sample scores from the species data, which makes it possible to plot sample scores as variation around the class centroids (tested environmental variable). To test the effects of soil treatment and plant origin, these treatments were coded as dummy variables and used as environmental data in the RDA analysis. In addition, to the RDA analysis we performed a one-way Analysis of Similarities (ANOSIM) using PAST (PAlaeontological STatistics version 2.06, Hammer, University of Oslo 1999-2011), which is a non-parametric test of significant differences between groups based on comparing distances between groups with distances within groups (Clarke, 1993) using Jaccard as distance measure running 10000 permutations. The distances are converted to ranks. Pairwise ANOSIMs between all pairs of groups (using 4 groups: native own soil, native control soil, range-expander own soil, range-expander control soil) are provided as post-hoc tests using x = 0.05 as significance cutoff.

To analyze the differences in number of fusaria copies per µl sample between soil treatments, origin and between plant species within origin, we used a nested ANOVA. All variables were considered fixed. To analyze the differences in amounts of ergosterol between soil treatment, origin and between plant species within origin, we used a similar nested ANOVA. All variables were considered fixed, including species, because we used all species that were available in our experimental site (Engelkes *et al.*, 2008). In order to test for a relationship between the distance of RDA scores in own and control soil on the first axis and total dry weight biomass difference between own and control soil treatments of the plants, a Spearman rank order correlation was performed with the species as replicate units. All univariate statistical analyses were carried out in STATISTICA 9 (StatSoft, Inc. 2009). To improve normality and homogeneity of variances of residuals among groups

defined by the statistical models, 18S rRNA gene copy number per µl sample and ergosterol values were ln(x+1)-transformed prior to analysis.

Results

Profiling of bacteria, fungi, arbuscular mycorrhizal fungi (AMF) and fusaria communities showed variation that could be explained, at least in part, by plant genus (Table S1, Figure 1). However, there was no further effect of plant species (Table S1). For all four groups of microbes the separation on genus level was significant, but the visual separation for general bacteria and fungi was more apparent than for AMF and fusaria (Figure 1). In addition to the expected differences among genera, there were significant effects of soil treatment (own vs. control soil) on the community structures of bacteria, fungi and fusaria (Table 2). However, there were no differences in the AMF-communities between own and control soils. The DGGE profiles did not reveal an effect of plant origin irrespective of the microbial groups examined (Table 2). However, there were significant interactions between soil conditioning and plant origin for bacteria, fungi and AMF, but not for fusaria (Table 2). The bacterial community in own versus control soils differed more for natives than range expanders (Figure 2). Although less obvious, differences in fungal communities between own and control soils were also greater in natives than in range expanders (Figure 2). However for fusaria, range expanders showed greater differences between own versus control soils than natives, whereas for the AMF, similar communities developed in the own soil treatments compared to the controls (Figure 2). The variation among individual AMF-samples in the individual plant species was considerably greater than in the other microbial groups (Figure 2). There was no correlation between differences in RDA scores of soil microbial community and plant biomass when comparing own and control soils: bacteria (n = 15; R^2 = 0.30; P = 0.277), fungi (n = 15; R^2 = -0.35; P = 0.201), AMF (n = 15; R^2 = -0.19; P = 0.499) and fusaria (n = 15; R^2 = -0.17; P = 0.541) (data not shown). Thus, these differences in microbial community composition are not indicative for general soil feedback effects.

According to the ANOSIM for the bacteria, the communities within groups (native control soil, native own soil, range-expander control soil, range-expander own soil) were more similar than between groups (R = 0.0514; P = 0.002). The similarity of own soil in native plants differed significantly from own and control soil in range-expanders (control soil: P = 0.0011; own soil: P = 0.0043). Moreover, the control soil of native and range expanding plants differed significantly (P = 0.0417), whereas there was no difference between control soil of natives and own soil in the range expanders (P = 0.1534) (Figure 2). The fungi (R = -0.018; P = 0.875), AMF-community (R = 0.0152; P = 0.146) and fusaria (R = -0.001; P = 0.485) did not show similarity among the four plant origin x soil conditioning combinations when using ANOSIM.
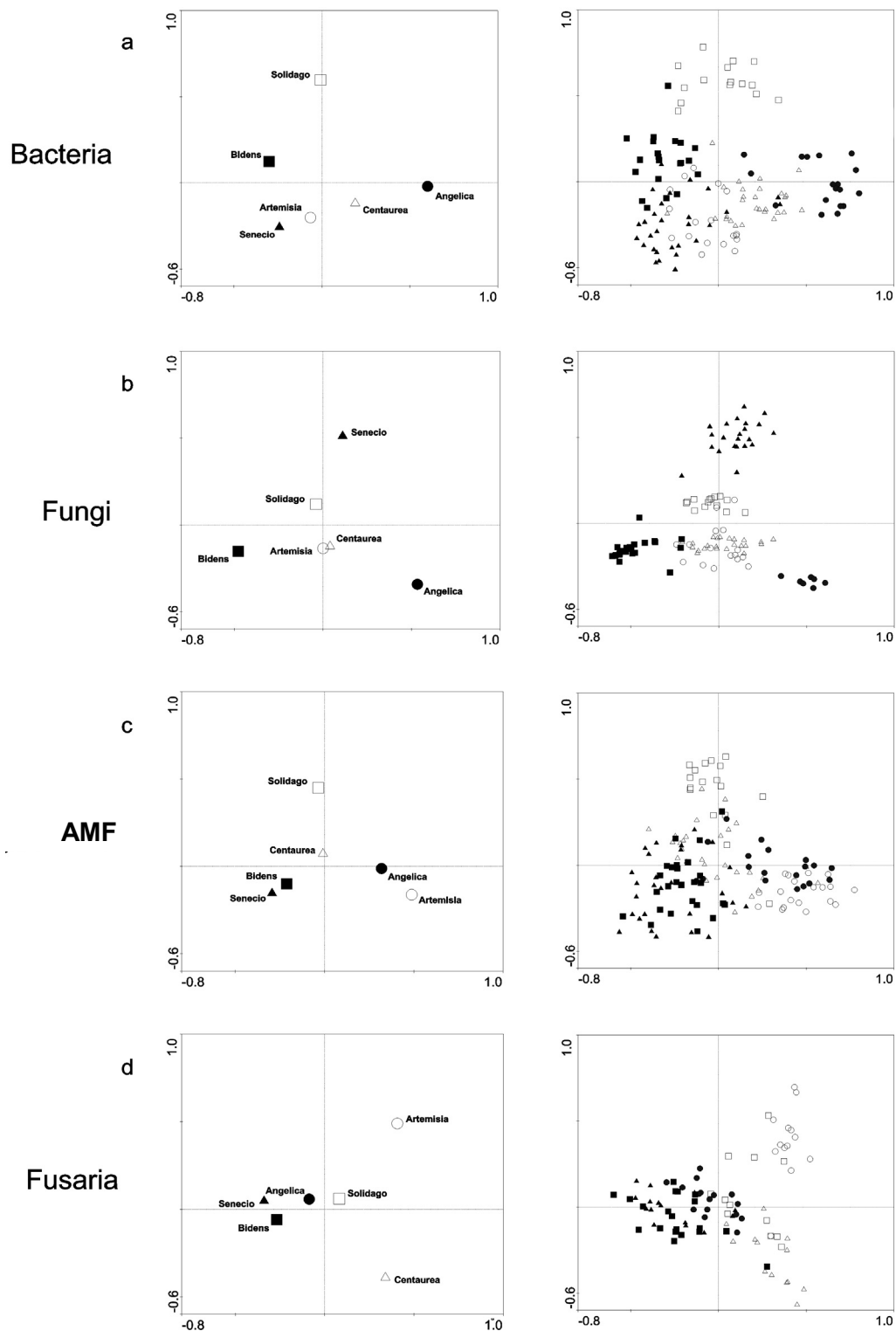
Figure 1 RDA ordination plots of DGGE banding patterns (left panels) with species plotted as nominal environmental variables in a scatter plot. The first and second RDA axes are plotted on the x and y axes, respectively. The right panels show the variation of the individual sample scores around their class centroids (species) from the left panel in a similar RDA ordination scatter plot. Symbols of samples in the right panels equal those of class centroids in the left panels: *Angelica* (filled circle), *Bidens* (filled square), *Senecio* (filled up-triangle), *Artemisia* (open circle), *Solidago* (open square) and *Centaurea* (open up-triangle). Panels show (a) bacteria; (b) fungi; (c) AMF (d) fusaria.

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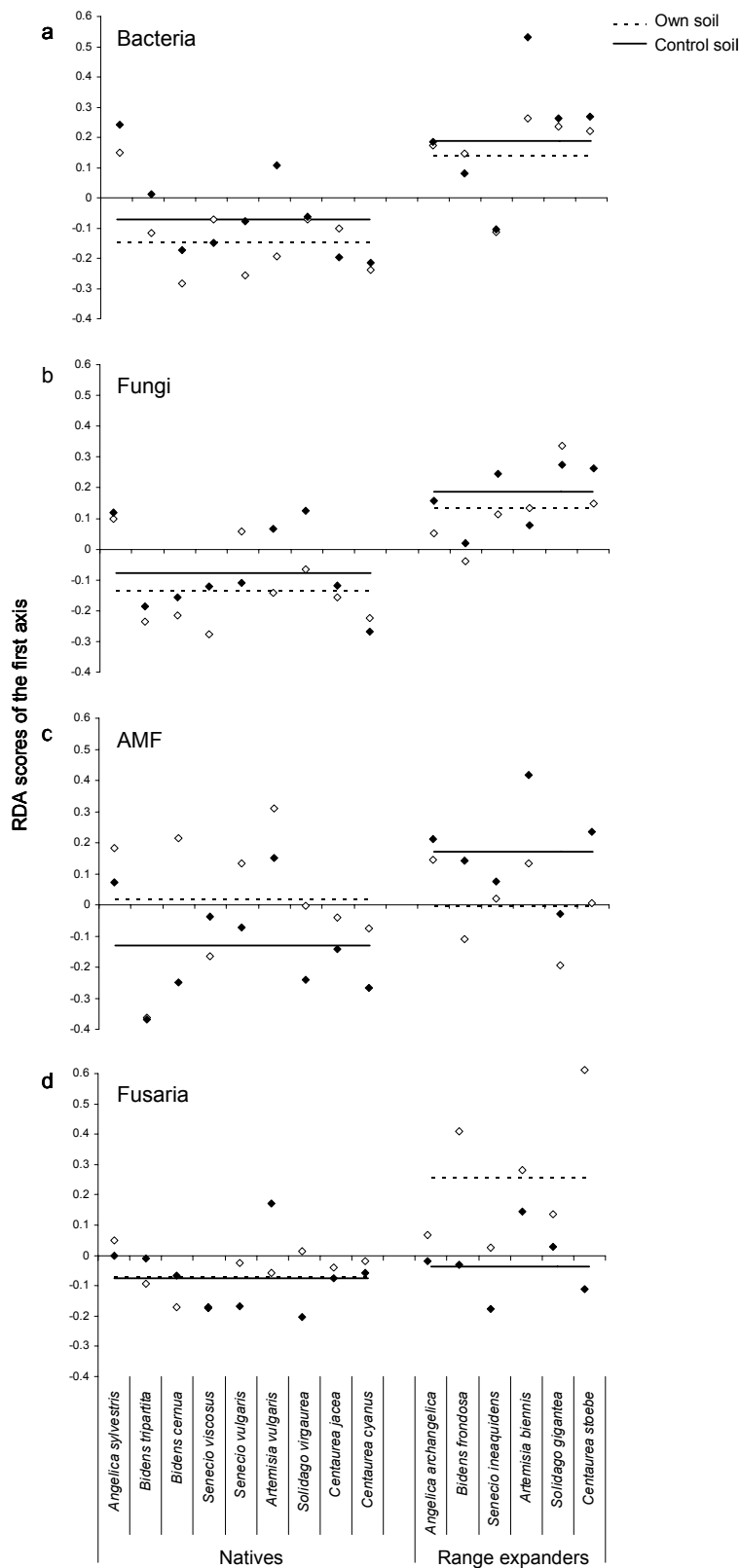
Figure 2 RDA centroid scores of the nominal environmental variables on the first RDA axis shown for native and range expanding plants. The RDA scores of the individual samples projected around their class centroids (soil treatment) on the first RDA axis are plotted as 'own soil' (open diamond) and 'control soil' (filled diamond) for each native and range-expanding plant species. The distance between own- and control soil lines indicates the average difference between their soil communities. Panels show (a) bacteria; (b) fungi; (c) AMF (d) fusaria.

Table 2 Results of the analyses using RDA and permutation tests showing the significance of the explanatory variables rhizosphere bacteria, fungi, arbuscular mycorhizal fungi (AMF) and fusaria as affected by soil (own versus control soil), plant origin (range expanding versus phylogenetically related native) and their interaction.

Explanatory variables	Covariables		Bacteria	Fungi	AMF	Fusaria
Soil treatment O, C	none	% expl. 1st axis	0.8	0.8	0.7	1.0
		r 1st axis	0.483	0.515	0.461	0.455
		F ratio	1.25	0.94	1.00	1.47
		P	0.004	0.006	0.104	0.008
Origin RE, N	Genus 1, 2, 3, 4, 5 & 6	% expl. 1st axis	1.6	1.5	0.7	1.0
		r 1st axis	0.658	0.684	0.533	0.566
		F ratio	3.17	2.54	1.29	1.85
		P	0.420	0.544	0.964	0.932
Soil treatment x Origin	All species, O, C	% expl. 1st axis	0.8	0.7	0.9	0.9
		r 1st axis	0.552	0.714	0.570	0.598
		F ratio	1.75	1.32	1.70	1.82
		P	0.002	0.050	0.008	0.260

Data are centered by species. No standardization by samples was done. Explanatory variables: are environmental variables in CANOCO terminology. % expl.1st axis: percentage of species variability explained by the first ordination axis, a measure of the explanatory power of the variables. r 1st axis: species-environment correlation on the first axis. F ratio: the F ratio statistics for the test on the trace. P: corresponding probability value obtained by the Monte Carlo permutation test using 499 random permutations. O: own soil treatment; C: control soil treatment; RE: Range Expander; N: Native.
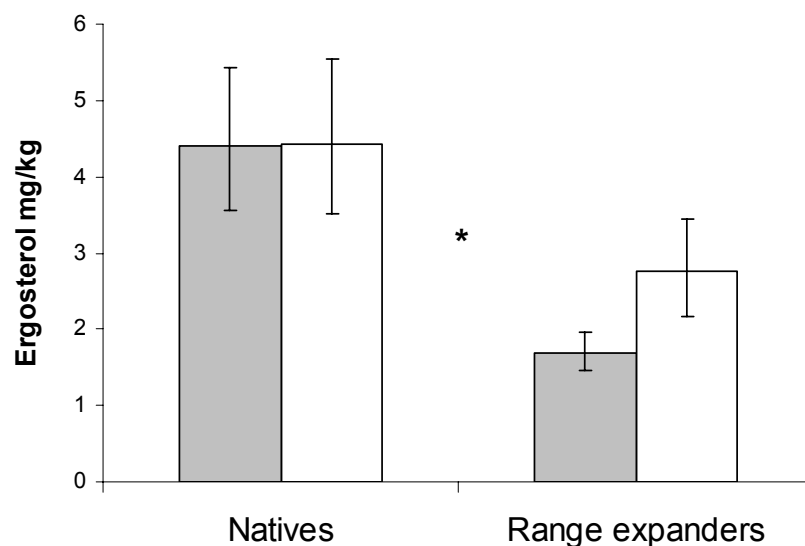

Figure 3 Ergosterol (mg.kg-1) in roots and attached rhizosphere soil in native and range-expanding plants after growing in own soil (white bar) and control soil (grey bar). Bars show back-transformed means ± 1 s.e. of ln(x+1)-transformed data. ∗ indicates significant differences.
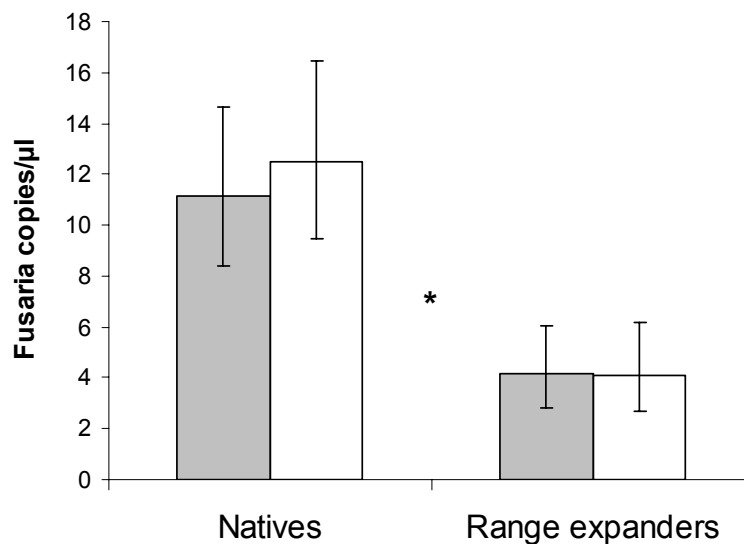
Figure 4 Number of fusaria genome copies per µl sample in native and range-expanding plants. Both plant origins have two treatments: own soil (white bar) and control soil (grey bar). Bars show back-transformed means ± 1 s.e. of ln(x+1)-transformed data. ∗ indicates significant differences.

Native plant rhizopsheres had higher fungal biomass than range expanders as judged by ergosterol measurement ($F_{1,83}$ = 31.79; P < 0.001) (Figure 3). There was no difference in fungal biomass between own and control soils nor for natives neither for range expanders ($F_{1,83}$ = 2.62; P = 0.11) (origin x soil interaction) (Figure 3).

Real-time PCR data revealed that the number of fusaria genome copies was significantly lower in the rhizosphere of range expanders than natives ($F_{1,108}$ = 17.81; P < 0.05) (Figure 4). Within plant origin, there were no differences in the number of genome copies between own and control soil.

Discussion

In the plant-soil feedback experiment, native plants on average had less shoot and root biomass in own than in control soil, whereas phylogenetically related range expanders did not show such reduction (Engelkes *et al.*, 2008). In the present study, we found that communities of bacteria, fungi, AMF and fusaria in the rhizosphere of these 15 plant species were influenced by plant genus, but opposite to expected (Kowalchuk *et al.*, 2002), there were no plant species-specific effects. Most likely, the differences between plant species of the same genus in our experimental setting were too subtle to be picked up by fingerprinting techniques such as DGGE. However, the effects of plant genus on the rhizosphere community supports the idea that the composition of the plant community can be a driver of the microbial rhizosphere community (Marschner *et al.*, 2001; Kowalchuk *et al.*, 2002; Carney & Matson, 2005; Hartmann *et al.*, 2009).

There were significant effects of own versus control soil on microbial community composition of bacteria, fungi and fusaria, yet there were no such effects

observed for AMF-communities. This supports the view that AMF have low species-specificity, when compared to e.g. soil pathogens (Eom *et al.*, 2000). DGGE did not reveal any effect of plant origin on any of the microbial groups. However, there were significant interactions between soil conditioning and plant origin for bacteria, fungi and AMF due to significant soil treatment differences between origins. Nevertheless, interaction effects only explained a relatively small amount of variation, which might be a consequence of using binary data which usually results in a lower multivariate fit (Legendre & Legendre, 1998). Minor differences in the soil community may result in only modest though detectable biomass differences when plants grow individually in a soil-feedback study (Kardol *et al.*, 2007). However, the same modest biomass differences could have large effects when plants grow under high competition (Weiner, 1985; Vilà & Weiner, 2004).

The ANOSIM results gave a slightly different outcome than the RDA analysis. ANOSIM is a robust non-parametric test that is not able to detect very small differences as RDA does. Also, we were not able to program the hierarchical structure of the design of the experiment in the ANOSIM, which also explains why some differences detected in the RDA analysis were not detected in the ANOSIM. Therefore, the differences we do detect with ANOSIM should be very robust. The bacterial communities within groups (native plants on control soil, on own soil, range-expanders on control soil, on own soil) were more similar than between groups, which is consistent with the findings based on the RDA-analysis displayed in Figure 2. The similarity in own soils of native plants differed significantly from own and control soil of range-expanders. Moreover, the rhizosphere bacterial communities of native and exotic plants in control soil differed significantly from each other. However, the bacterial community in control soil of native plants did not differ from own soil in the range expanders (Figure 2). Therefore, native plants develop the most specific soil community in soil in which they have been grown before. This seems consistent with studies that have reported intra-specific plant-soil feedback effects (Newsham *et al.*, 1995; Packer & Clay, 2000; Mitchell & Power, 2003; Sanon *et al.*, 2009). Such approaches may also be helpful in further unraveling why plant-soil feedback effects may operate more strongly on rare plants than on dominants (Klironomos, 2002). The strong effect of native plants on microbial communities in their own soils also supports the view of 'home-field advantage' (Gholz *et al.*, 2000; Chapman & Koch, 2007; Ayres *et al.*, 2009; Pregitzer *et al.*, 2010) on decomposition and nutrient cycling.

The non-parametric ANOSIM analysis did not reveal treatment differences for fungi, AMF and fusaria. The patterns in the fungi and AMF might not be robust enough to be picked up by ANOSIM due to the lower species-specificity in AMF. For fusaria, spores might have been absent from the soil inoculum community, since in a large number of samples no PCR product was recovered. This makes the analysis of fingerprinting data of fusaria less reliable, since the translation to binary data inflates small differences in the dataset.

There was no correlation of distances between the RDA scores in own and control soils of bacteria, fungi, AMF and fusaria and the biomass difference between own and control soil. There was also no direct correlation between the biomass differences of the soil treatments and the differences in the DGGE profiles. Therefore, the determination of any causal linkages between plant growth reduction and rhizosphere microbial community composition may require more subtle detection techniques than general profiling methods as DGGE.

We found more ergosterol in roots and rhizosphere soils of native plants than of range expanders. Although ergosterol cannot distinguish between living and dead fungi (Mille-Lindblom *et al.*, 2004; Zhao *et al.*, 2005), our ergosterol data suggest that range expanders had less fungal hyphal biomass in and around their roots than native plants. Ergosterol measurements provide no information on species composition or ecology of fungi, so that this biomarker may have originated from saprophytes, beneficials, or pathogens. There were no differences between control and own soils.

Real-time PCR data of *Fusarium* spp. revealed that genome copies were substantially fewer in the rhizosphere of range expanders than of native plants. Although this would suggest that range expanders are less exposed to potential pathogenic fungi, there were no differences in the number of genome copies in own soil and control soil within plant origin. When fusaria would have been involved in causing negative plant-soil feedback effects of native species, we would have expected different number of gene copies between own and control soil of native plants.

We conclude that range-expanding plants have a different microbial community structure than native plants, which is in line with our previous observations that the range expanders had the least negative soil feedback. The community effects were significant for bacteria, but not for fungi, arbuscular mycorrhizal fungi and fusaria. Range-expanding plant species had less (non-mycorrhizal) fungal hyphal biomass and fewer fusaria genome copies than related native plant species. However, our results did not yield evidence for a causal link between rhizosphere microbial community composition and plant-soil feedback effects at this level of detection.

Acknowledgements

Supplementary Information

Table S1 Results of the analyses using RDA and permutation tests showing the significance of the explanatory variables rhizosphere bacteria, fungi, arbuscular mycorhizal fungi (AMF) and fusaria as affected by plant species and plant genus.

Explanatory variables	Covariables		Bacteria	Fungi	AMF	Fusaria
Species	none	% expl. 1st axis	9.0	9.0	7.9	8.7
		r 1st axis	0.890	0.945	0.851	0.884
		F ratio	6.75	6.26	3.88	5.11
		P	1.000	1.000	1.000	1.000
Genus 1, 2, 3, 4, 5 & 6	none	% expl. 1st axis	8.6	8.7	7.2	8.4
		r 1st axis	0.873	0.932	0.827	0.878
		F ratio	10.61	10.21	5.85	7.91
		P	0.002	0.002	0.002	0.002

Data are centered by species. No standardization by samples was done. *Explanatory variables*: are environmental variables in CANOCO terminology. *% expl.1st axis*: percentage of species variability explained by the first ordination axis, a measure of the explanatory power of the variables. *r 1st axis*: species-environment correlation on the first axis. *F ratio*: the *F* ratio statistics for the test on the trace. *P*: corresponding probability value obtained by the Monte Carlo permutation test using 499 random permutations.

Plant species selection, seed germination and soil collection

We used four criteria for our selection: (1) exotic range expanding plants should have established in the Netherlands in the 20th century, (2) they should have increased in national grid cell abundance in the last decades of the 20th century in order to have been able to respond positively to climate warming (Tamis, 2005), (3) they should have related native species in the same genus and (4) all native and range expanding plant species occur in the same habitat (riparian areas in the Rhine Delta).

Five out of the six exotic plant species selected belonged to the family *Asteraceae* (*Bidens frondosa, Senecio inaquidens, Artemisia biennis, Solidago gigantea* and *Centaurea stoebe)* and one to the *Apiaceae* (*Angelica archangelica*). We grouped the exotic plants with nine native plant species (*Bidens cernua, Bidens tripartita, Senecio viscosus, Senecio vulgaris, Artemisia vulgaris, Solidago virgaurea, Centaurea cyanus, Centaurea jacea* and *Angelica sylvestris*). Of those plant species, we collected seeds from the field or, in a few cases, purchased seeds through a specialized seed supplier who collects seeds from local plant populations.

Surface sterilized seeds (1% hypochlorid solution for several minutes and then rinsing by demineralized water) were germinated on glass beads with demineralized water at a day of 14 hrs at 20°C and a night of 10 hrs at 10°C for species that flower in early season. A day of 16 hrs at 25°C and a night of 8 hrs at 15°C was used for species that flower in late season. Seedlings were placed at 4°C in light until all species had germinated and the experiment could start. After transplantation, dead seedlings were replaced until the third week of the experiment.

We collected soil from five random sites in the Millingerwaard (the Netherlands; 51°87′ N, 6°01′ E), a nature reserve in the Gelderse Poort region where all range expanding and related native plant species co-occur. The soil samples were homogenized, as we were not interested in spatial variation in the field, and inoculated into a sterilized sandy loam soil from Mossel, Planken Wambuis (52°06′ N, 5°75′ E). The soil sterilization was carried out by gamma radiation (25 kGray), which eliminated all soil biota (van der Putten *et al.*, 2007a)

Details experimental conditions

Phase I: soil conditioning. Each pot received four seedlings to promote soil conditioning and the experiment was carried out in a greenhouse under controlled conditions (60% RH, day: 21 ± 2°C; night 16 ± 2°C). Additional light was provided by metal halide lamps (225 μmol $^{-1}$ m^{-2} PAR) to ensure a minimum light intensity during daytime. Plants were provided with demineralized water every second day to compensate for water uptake and evapotranspiration. Every week, initial soil moisture level was reset by weighing. In order to prevent plants from nutrient depletion, Hoagland solution was added at a rate of 25 ml of 0.5 strength week^{-1}, which is a dosage that does not prevent the establishment of arbuscular mycorrhizal fungi (van der Putten *et al.*, 2007a).

Phase II: soil feedback. We established five replicates with own and five with control soils (resulting in 150 pots): each replicate was made from a separate replicate from the soil conditioning phase. We planted three seedlings per pot. Water, light and nutrient conditions were supplied as in phase I, except that 10 ml of 0.5 strength Hoagland solution was added on a weekly basis because the pots were smaller, there were fewer plants and there was less water loss from the soil surface compared to the 4 L pots.

Ergosterol measurements

Ergosterol was measured using a Dionex HPLC equipped with a C 18 reverse-phase column (All-sphere ODS-2 5 μ, 250 × 4.6 mm; Alltech, Deerfeld, USA) and a UV detector set a 282 nm. The column temperature was kept at 30°C. Methanol (HPLC grade) was used as the mobile phase at a flow rate of 1.5 ml/min. At these conditions, the retention time of ergosterol was 12 min. Sample size for injection was 20 μl. Pure ergosterol (purity >90%; Sigma Chemicals, St Louis, USA) was used for constructing a standard curve.

Chapter 6

SOIL AND PLANT COMMUNITY EFFECTS ON THE PERFORMANCE OF EXOTIC RANGE-EXPANDING PLANT SPECIES AND PHYLOGENETICALLY RELATED NATIVES

Elly Morriën & Wim H. van der Putten

Abstract

It has been acknowledged that climate warming induced range expansion of plants can result in a release from soil-borne pathogen activity in the new range. However, little is known about the advantage that this may bring to range-expanding plant species in their new habitat. Here, we compare early establishment of range-expanding exotic plant species and phylogenetically related plants that are native in the invaded habitats. In a greenhouse, we grew five range-expanding plant species and five related natives in sterilized and non-sterile soils from the invaded habitat both alone and in a plant community of species present in the invaded habitat. In the field, we grew the same plant species in plots with sparse versus dense plant communities. Opposite to range expanders, native plant species are exposed to soil-borne pathogen activity, so that we expected range expanders to perform better than natives in a competitive environment with soil biota. In the greenhouse, both native and range-expanding plant species benefited from low competition from the surrounding plant community irrespective of the presence or absence of a soil community. In the field, where we did not experimentally change the soil biotic conditions, most plants performed better in sparsely vegetated plots than in densely vegetated plots. We conclude that for the initial establishment, competition by the surrounding plant community is equally important for range-expanding plant species and phylogenetically related natives. If there are benefits for range-expanding plant species of being released from soil-borne pathogenic activity, our results suggest that these benefits will become important only after initial establishment when natives will develop soil pathogenicity and range expanders not.

Introduction

One of the consequences of the current climate warming is that many plant species shift ranges to higher latitudes (Bakkenes *et al.*, 2002; Walther *et al.*, 2002; Parmesan & Yohe, 2003). As a result, plant species from lower latitude areas currently invade plant communities in previously cooler climate regions (Williamson & Fitter, 1996; Dukes & Mooney, 1999; Kolar & Lodge, 2001). Rapid range expansions may result into disruptions of trophic interactions, because not all species have equal dispersal capacities. Especially soil biota disperses slower than most plant species (Berg *et al.*, 2010). This may influence the net outcomes of interactions between plants and soil biota, as range-expanding plant species can benefit from release from soil-borne enemies (van Grunsven *et al.*, 2007, 2010; Engelkes *et al.*, 2008; Kulmatiski & Beard, 2011). On the other hand, symbiotic mutualists that are supposed to be less specialized (Richardson *et al.*, 2000) may establish symbiotic relationships with range-expanding plant species in their new range. Little is known about how these altered balances between enemies and symbiotic mutualists in the soil may enhance competitiveness of exotic range-expanding plants in their new habitats.

Many exotic plant species establish in ruderal areas that are frequently disturbed by natural processes (e.g. flooding by rivers) or human activities (e.g. construction works or soil tillage). As a result of these disturbances, natural succession is often re-set, which results in relatively sparse vegetation cover with low competition pressure from other plant species (Burke & Grime, 1996; Hierro *et al.*, 2006). Once established, competition with the surrounding vegetation for light, water and nutrients may intensify (Fargione *et al.*, 2003; Dietz & Edwards, 2006). Non-disturbed plant communities that are more densely vegetated will provide fewer opportunities for exotic species to invade, which can be considered as 'biotic resistance' (Maron & Vila, 2001; Parker & Hay, 2005). Although this phenomenon rarely enables communities to resist invasion, it constrains the abundance of invasive species once they have successfully established (Levine *et al.*, 2004).

In summary, the mechanisms that can promote establishment of intercontinental invaders may include low levels of competition on disturbed sites, reduced exposure to natural enemies, and enhanced net benefits from low-specific symbionts (Colautti *et al.*, 2004). The exposure of range-expanding plants to biotic interactions in the new range is expected to differ from that of phylogenetically related native species, because of an altered balance between soil-borne enemies and mutualists. Therefore, the question that we address in the present study is if range-expanding plant species may be better competitors with the native vegetation than phylogenetically related natives and whether this may depend on the presence or absence of soil biota.

We tested the hypothesis that range-expanding plant species establish better under competition with native vegetation than phylogenetically related natives, because exotic plants benefit from less negative interactions with the soil community than natives. Thus far, benefits from reduced negative interactions with the soil

community in range-expanding plants have been shown to work effectively in monocultures (van Grunsven *et al.*, 2007; Engelkes *et al.*, 2008). We expected that competition with the surrounding plant community would enlarge these benefits for the range expanders (Weiner, 1985; Vila & Weiner, 2004) when exposed to soil communities from the invaded range. In order to test this hypothesis, we grew five range-expanding plants and five phylogenetically related natives, all currently occurring in the same habitat, in a greenhouse and in the field. In the greenhouse, we tested the effects of soil community on both native and range-expanding plant species without and with interspecific competition. In the field, we planted the same range expanding and phylogenetically related native plant species both in densely and sparsely vegetated plots in order to determine if range-expanding plants perform better under competition than phylogenetically related natives. We discuss our results in relation to the role of vegetation density and feedback effects from soil biota during early establishment of range-expanding plant species.

Material and Methods

Plant seedling selection and seedling growth
We selected recently introduced range-expanding plant species and phylogenetically related natives using the National Standard List of the Dutch flora using four criteria: (1) range-expanding plants should have established in the Netherlands in the 20th century, and (2) they should have increased in national grid cell abundance in the last decades of the 20th century in order to have been able to respond positively to climate warming (Tamis, 2005), (3) they should have a closely related native species (within the same genus, except one species of which we took a relative at the family level with a comparable ecology) and (4) all native and range-expanding plant species occur in the same habitat (we used riparian areas in the Rhine Delta where many range-expanding plant species co-occur with related natives). These four criteria enabled us to produce a phylogenetically controlled comparison of native and recently invading range-expanding plant species (Agrawal *et al.*, 2005; Funk & Vitousek, 2007) sharing the same habitat. Of those plant species, we collected seeds from the field or, in a few cases, purchased seeds through a specialized seed supplier who collects seeds from local plant populations.

Three out of the five range-expanding plant species belonged to the family *Asteraceae* (*Senecio inaequidens*, *Artemisia biennis*, and *Centaurea stoebe*) and two to the *Brassicaceae* (crucifers) (*Bunias orientalis* and *Rorippa austriaca*). Four range-expanding plant species originated from Eurasia: *A. biennis* (North-Asia), *C. stoebe* (Central Europe), *B. orientalis* (Central Europe) and *R. austriaca* (Central Europe). One range expander originated from another continent: *S. inaequidens* (South-Africa). We included five related native plant species (*Jacobaea vulgaris* (previously known as *Senecio jacobaea*), *Artemisia vulgaris*, *Centaurea jacea*, *Sinapis arvensis* and *Rorippa sylvestris*).

For the greenhouse experiment all seeds were surface sterilized prior to germination using 100 ml diluted Loda Bleech (1% hypochlorid solution) for several minutes and then rinsed by demineralized water. Seeds were germinated on glass beads with demineralized water at conditions of 8-16 hrs at 15-25°C night-day regime. Seedlings were placed at 4°C in light until all species had germinated and the experiment could start. After transplantation, dead seedlings were replaced until the second week of the experiment. For the field experiment seeds were germinated on trays with homogenized sterilized soil at conditions of 8-16 hrs at 15-25°C night-day regime. Seedlings were six weeks old when planted in the field and seedlings that had died in the field after transplantation were replanted until the fourth week of the experiment. All plant pairs were transplanted during a 2.5 weeks time interval and paired native and range-expanding plants were planted on the same day.

Greenhouse experiment

There were two soil treatments, sterilized field soil and sterilized field soil inoculated with living field soil. All soil originated from the Afferdense en Deestse Waarden, a riverine habitat in the Netherlands (51°89′ N, 5°64′ E). Per soil treatment we had pots sown with a background plant community of species that also occur in the field site and pots without such sown plant communities. We examined the responses of ten plant species to soil inoculation and plant community presence. Five species were range-expanders and five were phylogenetically related natives. We established ten replicates per plant species. Additionally, we established ten control pots with background plant community sown in sterilized soil and ten with inoculated field soil. This resulted in 2 soil types (sterilized and inoculated) x 2 backgrounds (with and without plant communities) x 2 plant origins (range expander and native) x 5 phylogenetic combinations x 10 replicates + 2 x 10 control pots = 420 pots. The pots were 1.3 L and each pot (except the 20 control pots) received one seedling of the target plant species.

After collecting the soil from the Afferdense en Deestse Waarden the samples were sieved to remove roots and stones. Then, 4/5th of the soil was sterilized by gamma irradiation (25 kGray), which eliminated all soil biota (van der Putten *et al.*, 2007). Half the pots were filled with 100% sterilized soil and the other half with a 5:1 mixture of sterilized soil and living field inoculum. Each pot contained 1665 gram of soil with a moisture content of 14% based on dry soil weight (w:w).

The background plant community consisted of 40% grasses (*Agrostis capillares, Festuca rubra* and *Phleum pratense*) and 60% forbs. The plant species in this plant community all occurred in the riverine habitat of the nature reserve the Afferdense en Deestse Waarden. In order to approach field densities of plants, 4 grams of seed mixture/m² was supplied. Within the 60% forbs, the total mixture contained 50% dominant forb species (*Plantago lanceolata, Glechoma hederacea, Trifolium pratense* and *Medicago lupulina*, 1016 mg seeds of each), 30% semi-dominant species (*Arctium lappa, Plantago major, Trifolium repens* and *Allium vineale*, 610 mg of seeds each) and 20% low abundant plant species (*Cirsium vulgare, Bellis perennis* and *Tripleurospermum*

maritimum, 542 mg seeds of each). Because some forbs had large seeds we added an extra amount of seeds for those species so that at least each of the 220 pots had a chance to receive one or several individuals. As a result, we added 1627 mg extra seeds of *Trifolium pratense*, 1709 mg extra seeds of *Arctium lappa*, and 2921 mg extra seeds of *Allium vineale* to the total seed mixture. After preparing the total seed mixture we distributed the amount of seeds equally over the 220 pots.

The pots were placed in a greenhouse under controlled conditions (60% RH, day: 21 ± 2°C; night 16 ± 2°C). Additional light was provided by metal halide lamps (225 μmol $^{-1}$ m^{-2} PAR) to ensure a minimum light intensity during the day. Plants were provided with demineralized water every second day to compensate for water uptake and evapotranspiration. Every week, initial soil moisture level was reset by weighing. We measured the direct effect of the soil community and background plant community on the focal plant species during seven weeks. After seven weeks all focal plants were harvested and shoot and root dry weights were determined separately. Roots were collected by water rinsing in a sieve and drying afterwards. The dry biomass of the background plant community was also determined separately for shoots and roots. For the shoots it was possible to distinguish between forbs and grasses, plants that were sown and plants that germinated from the seed bank that was present in the living field soil in the inoculated pots.

Field experiment

We established 10 plots each consisting of two subplots of 1.9 x 2.6 m with 70 cm space in between subplots. One subplot had dense riverine vegetation, whereas the other subplot had only sparse plant cover. Each of the subplots was divided into squares of 50 x 50 cm with 20 cm in between the squares. In five of these squares we planted the seedling of a range expander and in the other five squares we planted a related native species. All seedlings were planted in the centre of the square and seedlings were distributed across the subplots according to a random design. The ten plots in the field were considered as independent replicates. Thus, our experimental design was as follows: 2 plant cover densities (dense and sparse) x 2 plant origins (native and range expander) x 5 species x 10 replicates = 200 experimental squares.

The field site was situated in a riverine habitat in the nature reserve the Afferdense en Deestse Waarden, the Netherlands (51°89′ N, 5°64′ E). A fence excluded large vertebrate herbivores (mainly horses). The sparsely covered subplots were created because root cloth had covered these subplots after soil tillage of the entire field site in spring 2008. The densely vegetated subplots were not covered with root cloth after tillage and thus contained a one year old vegetation of natural riverine plant species. The field experiment was performed from June 16th until October 2nd 2009. Upon harvest we carefully removed the planted focal target plants including roots. We determined the shoot and root dry weights separately by water rinsing in a sieve and drying at 70°C for 72 hrs.

Data analyses
Greenhouse experiment: we analyzed the differences between plant origin, competition and soil sterilization using them as fixed factors in a full factorial ANOVA with plant species as replication unit. The effects of plant origin and soil sterilization on the background plant community were also analyzed using a full factorial ANOVA with fixed factors and plant species as replication unit. To improve normality and homogeneity of variances, shoot and root biomass of the focal plants as well as the root biomass of the background plant community were ln(x+1)-transformed prior to analysis. The shoot biomass of grasses versus forbs in the background community was analyzed using a full factorial ANOVA similar as for the total background vegetation.

Field experiment: we analyzed the differences between plant origin and plant cover densities of the plant community using a factorial randomized block design with block (plot) as random factor and plant origin and plant cover density as fixed factors. Because there were no significant effects of block (plot), we also analyzed the data using a full factorial ANOVA with plant origin and plant cover density as fixed factors and plant species as replication unit. All statistical analyses were carried out in STATISTICA 9 (StatSoft, Inc. 2009).

Results

Greenhouse experiment
There was a strong negative effect of the background plant community on the shoot biomass of focal range expanding and native plants ($F_{1,32} = 51.16$, $P < 0.001$) (Figure 1a). Shoot biomass of range-expanding focal plants did not differ from native focal plants ($F_{1,32} = 0.649$, $P = 0.426$) (Figure 1a), also not in combination with the effect of competition from the background plant community ($F_{1,32} = 0.029$, $P = 0.865$). Although all focal plants (range expanders and natives) showed a tendency to perform better when growing in the sterilized soils (Figure 1a), there was no significant difference with sterilized soils inoculated with non-sterile field soil ($F_{1,32} = 0.718$, $P = 0.403$). Shoot biomass reduction as a result of field soil inoculum was visible only in the monoculture pots without background plant community (Figure S1a). Root biomass of range expanding and native focal plants was also significantly reduced by competition from the background plant community ($F_{1,32} = 50.55$, $P <0.001$). However, the pattern in roots appeared to be weaker than in the shoots (Figure 1b). Similar as in the shoots there were no differences between root biomass of range expanders and native focal plants neither without ($F_{1,32} = 2.543$, $P = 0.121$) (Figure 1b) nor with competition from the background plant community ($F_{1,32} = 2.180$, $P = 0.150$). The trend that focal plants produced more root biomass in sterilized soils than in inoculated soils, was not significant ($F_{1,32} = 0.703$, $P = 0.408$) (Figure 1b). Soil effects on root biomass were visible only in the pots without background plant community (Figure S1b).
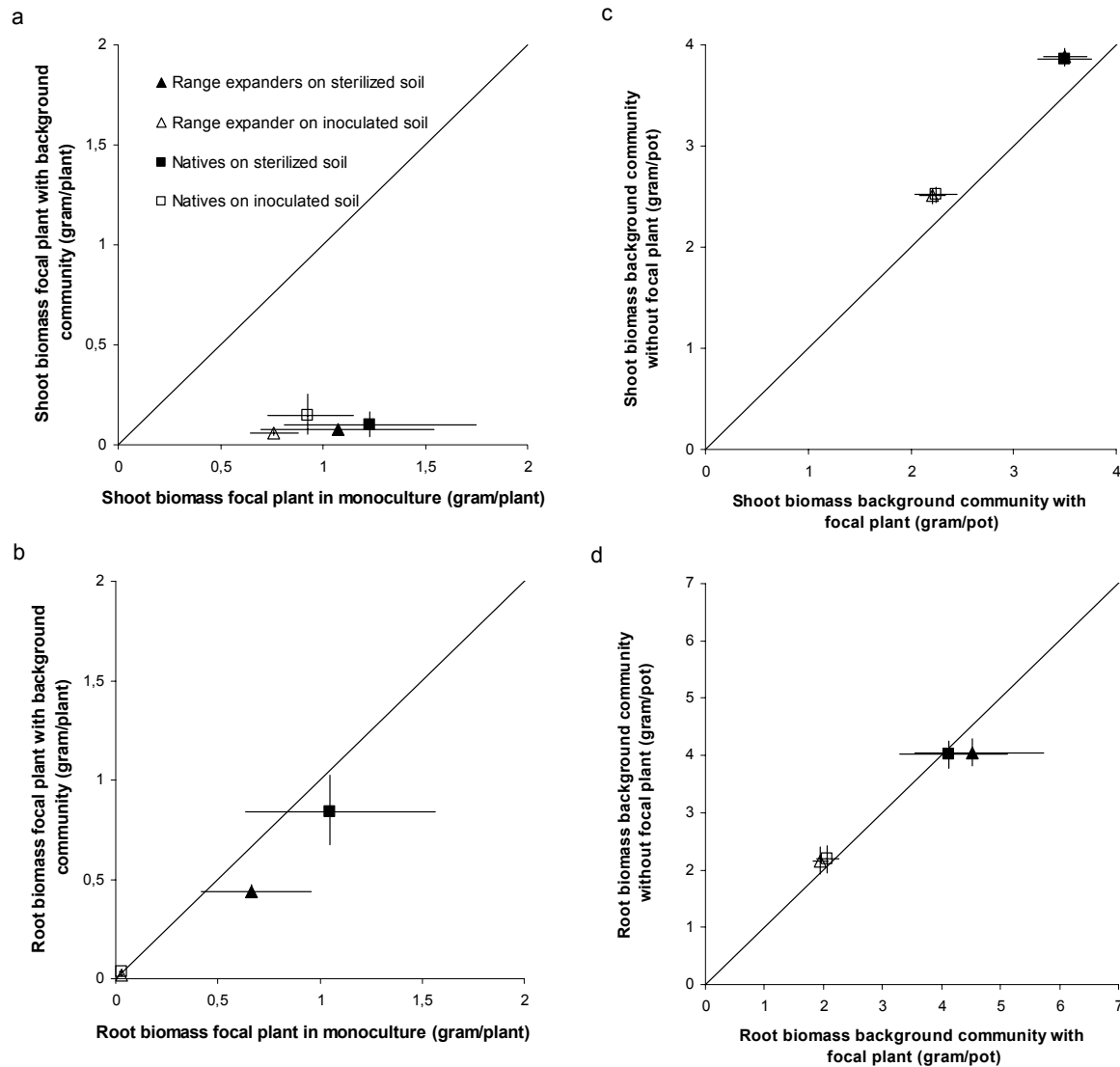
Figure 1 *Greenhouse experiment.* Focal plants: mean shoot (panel a) and root (panel b) biomass ± s.e. in sterilized and inoculated soils of range expanding and native focal plants in monoculture plotted against that of the focal plants in pots with background community. Background community: mean shoot (panel c) and root (panel d) biomass ± s.e. of the background community in sterilized and inoculated soils with focal plants of native and range-expanding origin against that of the background community without focal plants.

The shoot biomass of the background plant community was not affected by the range expanding or native focal plants ($F_{1,16} = 0.006$, $P = 0.937$) (Figure 1c). There was more background community shoot biomass when plants were growing in sterilized soils than in inoculated soils ($F_{1,16} = 37.87$, $P < 0.001$) (Figure 1c). In sterilized soils there was more grass biomass ($F_{1,16} = 89.07$, $P < 0.001$) and less forb biomass ($F_{1,16} = 56.83$, $P < 0.001$) than in inoculated soils (data not shown). The root biomass of the background plant community was not influenced by the focal range expanding or native plants ($F_{1,16} = 0.019$, $P = 0.893$) (Figure 1d). However, there was significantly more background community root biomass in sterilized than in inoculated soils ($F_{1,16} = 17.31$, $P < 0.001$) (Figure 1d).
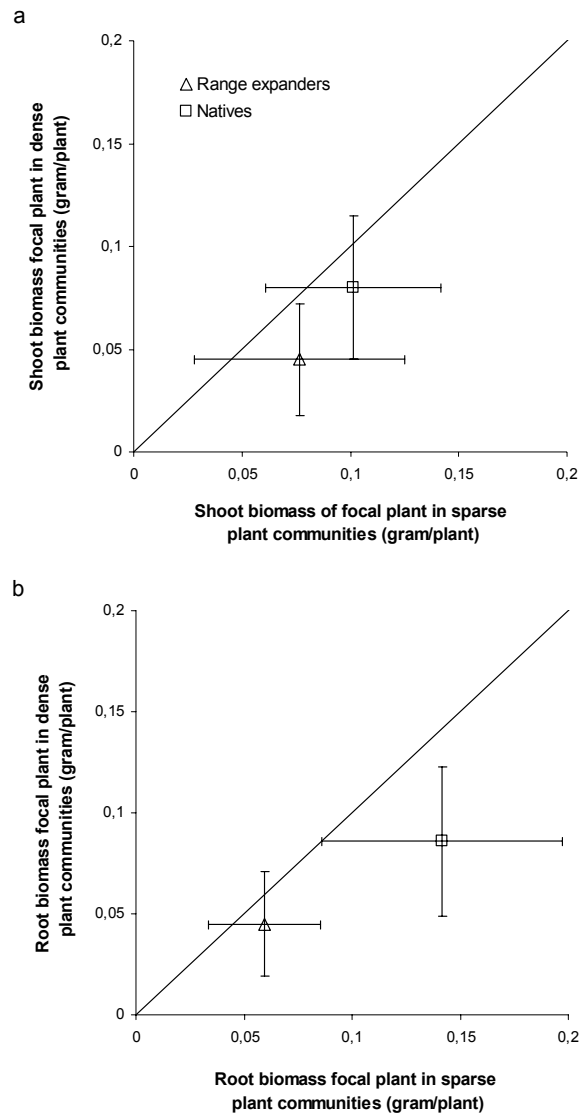
Figure 2 *Field experiment.* Mean shoot (panel a) and root (panel b) biomass ± s.e. of focal plants of range expanding and native origin in sparse plant communities against the mean biomass ± s.e. of the focal plants in dense plant communities.

Field experiment

In the field experiment there was no block effect for the shoot biomass of the focal plants ($F_{9,138}$ = 0.540, P = 0.844) and also not for their root biomass ($F_{9,138}$ = 1.460, P = 0.170). We therefore regarded the blocks (plots) as independent replicas in the field. There was no difference between shoot biomass of range expanding and native focal plant species ($F_{1,16}$ = 0.598, P = 0.451) (Figure 2a). Most natives and range expanders appeared to perform slightly better in sparse than in dense plant communities, but this was not significant ($F_{1,16}$ = 0.473, P = 0.501) (Figure 2a). Range-expanding focal plants did not benefit more from sparse plant communities than native focal plant species ($F_{1,16}$ = 0.018, P = 0.896) and they did not perform different from native focal plants in densely vegetated plant communities (Figure 2a, Figure S2a). Also for the

root biomass there was no significant difference between focal plants of range expanding and native origin ($F_{1,16}$ = 2.621, P = 0.125) (Figure 2b). All range expanders and natives tended to produce most root biomass in sparse plant communities, but this was not at all significant ($F_{1,16}$ = 0.855, P = 0.369) (Figure 2b). Similar as for the shoot biomass, there was not more root biomass of range-expanding focal plant species in sparse plant communities than native focal plants species ($F_{1,16}$ = 0.293, P = 0.595) (Figure 2b, Figure S2b). Responses in biomass between plant species within native-range expander plant pairs were quite diverse. *A. vulgaris* and *S. arvensis* are natives that performed relatively well compared to their range expanding counterparts, whereas the range expanders *S. inaequidens* and *R. austriaca* performed relatively well compared to their native counterparts (Figure S2).

Discussion

Release from belowground enemies has been proposed to act as one of the drivers of invasion success of exotic plant species (Colautti *et al.*, 2004; Levine *et al.*, 2006) and might play a role in range expander establishment. Based on their responses in plant-soil feedback experiments (van Grunsven *et al.*, 2007; Engelkes *et al.*, 2008), we had expected range-expanding plant species to perform better than related natives when competing with background plant communities in sterilized soil inoculated with non-sterilized field soil. In that soil the range expanders were expected to benefit relatively strongest from enemy release. However, in the greenhouse experiment there was a strong negative effect of competition from the background plant community on the shoots and the roots of both range-expanding and native plant species. Opposite to our expectations, soil inoculation effects were visible only in the monoculture pots without background plant community (Figure S1). Kardol *et al.* (2007) found that competition by background plant community enforced growth reduction effects caused by soil biota. However, they observed these enforcement effects only when the focal plants were early successional plant species and the background plant community consisted of later successional species, which are known to differ in plant-soil feedback effects (Kardol *et al.*, 2006). Since we grew plant species from the same successional stage in competition with each other, similar responses to soil biota of plant species from the same successional stage might explain why the effect of competition was more profound than the effect of the soil community.

Most native and range-expanding plant species benefitted from sterilized soil in the absence of competition. Effects of mobilization of soil nutrients will have been relatively minor, since the pots with inoculated soil also contained 4/5 of sterilized soil, which largely rules out confounding effects of nutrient availability on shoot and root biomass (Figure S1). *Centaurea* spp. however, appeared to be negatively influenced by the absence of a particular biotic soil component in the sterilized soil. Apparently, these species may have a net benefit from the soil community in a competition-free environment. Range expanding wild crucifers (*B. orientalis* and *R.*

austriaca) were expected to benefit from the enemy release effect, but not from symbiotic mutualistic relationships since they are non-mycorrhizal. However, the *B. orientalis-S. arvensis* and *Rorippa* spp. pairs reacted quite different regarding soil inoculation and competition from the background plant community (Figure S1).

The shoot and root biomass of the background plant community itself did not respond differently to growing with range expanding or native focal plant species. Therefore, our results do not point at a role of novel weapons (Callaway & Ridenour, 2004; Cappuccino & Arnason, 2006) of introduced plant species that may reduce the performance of native vegetation. There was more background community biomass in sterilized than in inoculated soils. This was mainly due to sterilized soils promoting grass biomass at the cost of forb biomass, which appears to be quite a repeatable response to soil sterilization (De Deyn *et al.*, 2004b).

In the field experiment the shoot and root biomass of range expanding and native focal plant species were not different. Most focal plants (range expanders and natives) appeared to produce more root biomass in sparse than in dense plant communities, but this effect was not significant. Range-expanding focal plants did not benefit more from sparse plant communities than native focal plant species in terms of shoot and root biomass (Figure 2, Figure S2). However, within plant pairs there were some species-specific differences in plant performance. The results of the field experiment are in line with the results of the greenhouse experiment: range-expanding plant species did not show a benefit of their lower sensitivity to soil-borne pathogens in the soil community compared to related natives.

We had expected that reduced competition from the background plant community would provide major benefits to all planted seedlings, which was not the case. This result may have been influenced by the warm and dry conditions during and after establishing the experiment. Under those conditions, bare soil appeared to dry out sooner than soil covered by vegetation, which also provided some shade thereby protecting the seedlings from desiccation. Thus, in extreme conditions the background plant community might even facilitate the establishment of both native and exotic plant species in regeneration niches.

Direct effects of sterilized versus inoculated soil affected native plants as much as range expanders. However, this experiment was not carried out as a test on soil feedback; our experiment only concerned the conditioning phase of a classic plant-soil feedback experiment. Previous studies have shown that range-expanding plant species, including a number of plants used for the present study, develop less negative feedback than related natives (van Grunsven *et al.*, 2007; Engelkes *et al.*, 2008). From that perspective, range-expanding plants perform similar to intercontinental exotics (Klironomos, 2002). The less negative plant-soil feedback of intercontinental exotics and thus range expanders, can be due to absence of soil-borne pathogens in the new range (Bever, 2003; Reinhart *et al.*, 2003), or to enhanced benefit from soil-borne mutualists (Reinhart & Callaway, 2006). However, there was no clear pattern in our data that the crucifer species, which are non-mycorrhizal, responded different from the other plant species.

In our study, we show that the background plant community can reduce the performance of both native and range-expanding plants, but that under dry conditions in the field the surrounding plants might as well facilitate by protecting against desiccation. In the greenhouse, there was no exclusive indirect benefit to range-expanding plants when growing in sterilized soil inoculated with non-sterilized field soil. This does not necessarily imply that there is no benefit to range-expanding plants from release from soil-borne pathogenic activity. During prolonged presence on local sites, plant-soil feedback may over time become more negative to natives than range expanders (Engelkes *et al.*, 2008; van Grunsven *et al.*, 2007). Since the legacies of these plant soil-feedback effects remain present in the field for longer time (Kulmatiski & Beard, 2011), they ultimately may shift the competitive outcome between range expanders and the surrounding plant community compared to related natives. However, in order to test that hypothesis, plant-soil feedback studies in the field need to be carried out during a number of subsequent growing seasons.

We conclude that during conditioning of the soil biota both range expanding and related native plant species may benefit from low competition and that the chances to invade a new spot is more species-specific than determined by the origin of the plant. The direct soil community effects that we tested by comparing growth in sterile versus inoculated soil were overruled by the competition effects from the background plant community. However, plant-soil feedback comparisons between range-expanders and phylogenetically related natives (van Grunsven *et al.*, 2007; Engelkes *et al.*, 2008) would suggest that over time the competitive strength of natives will reduce due to the build-up of net negative soil feedback, whereas the competitiveness of range expanders may remain, owing to their more neutral soil feedback effects.

Acknowledgements

Supplementary Information

Shoot and root biomass of individual plant species in greenhouse and field experiment.

Figure S1 *Greenhouse experiment*, mean shoot (panel a) and root (panel b) biomass ± s.e. of the focal plants in pairs. Natives positioned on the left and their range-expanding congeners on the right side in sterilized and inoculated soils with and without background plant community.
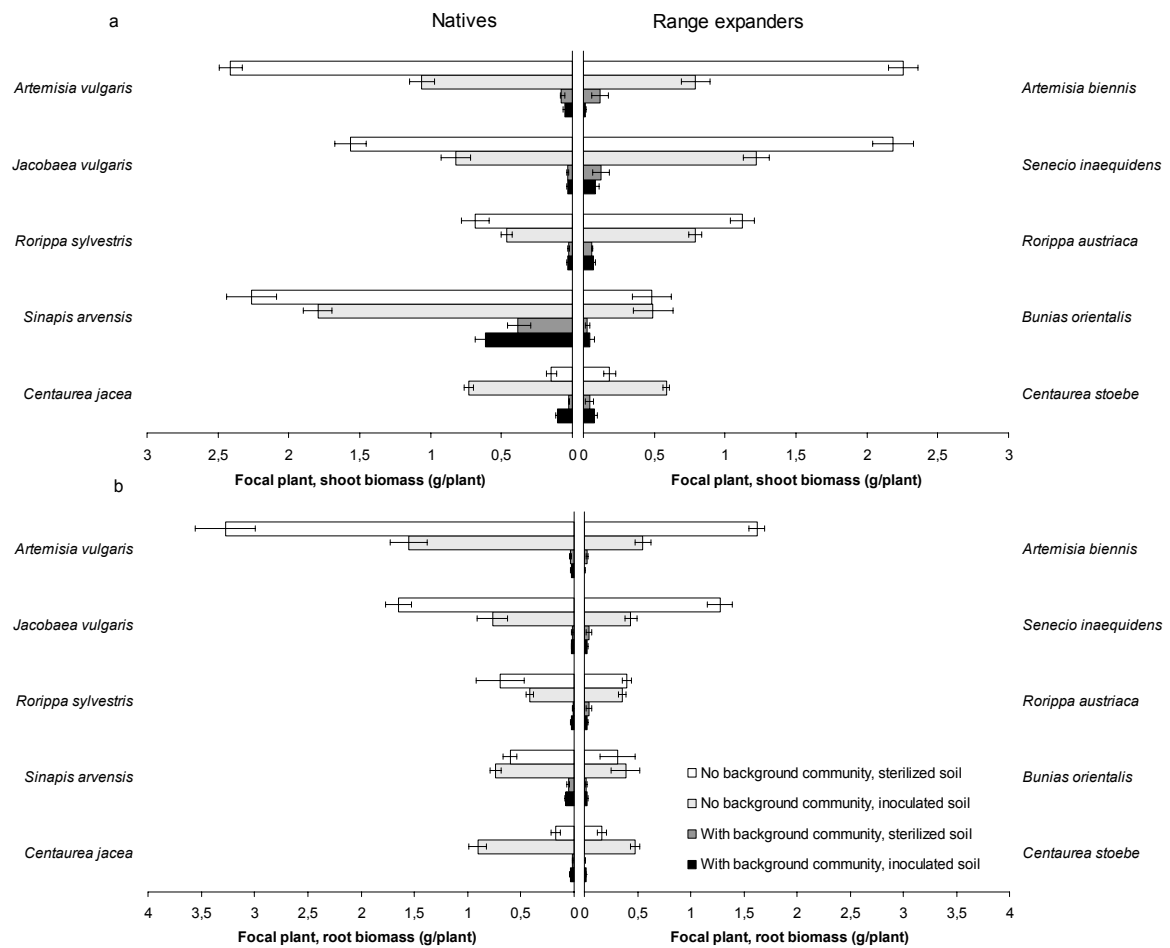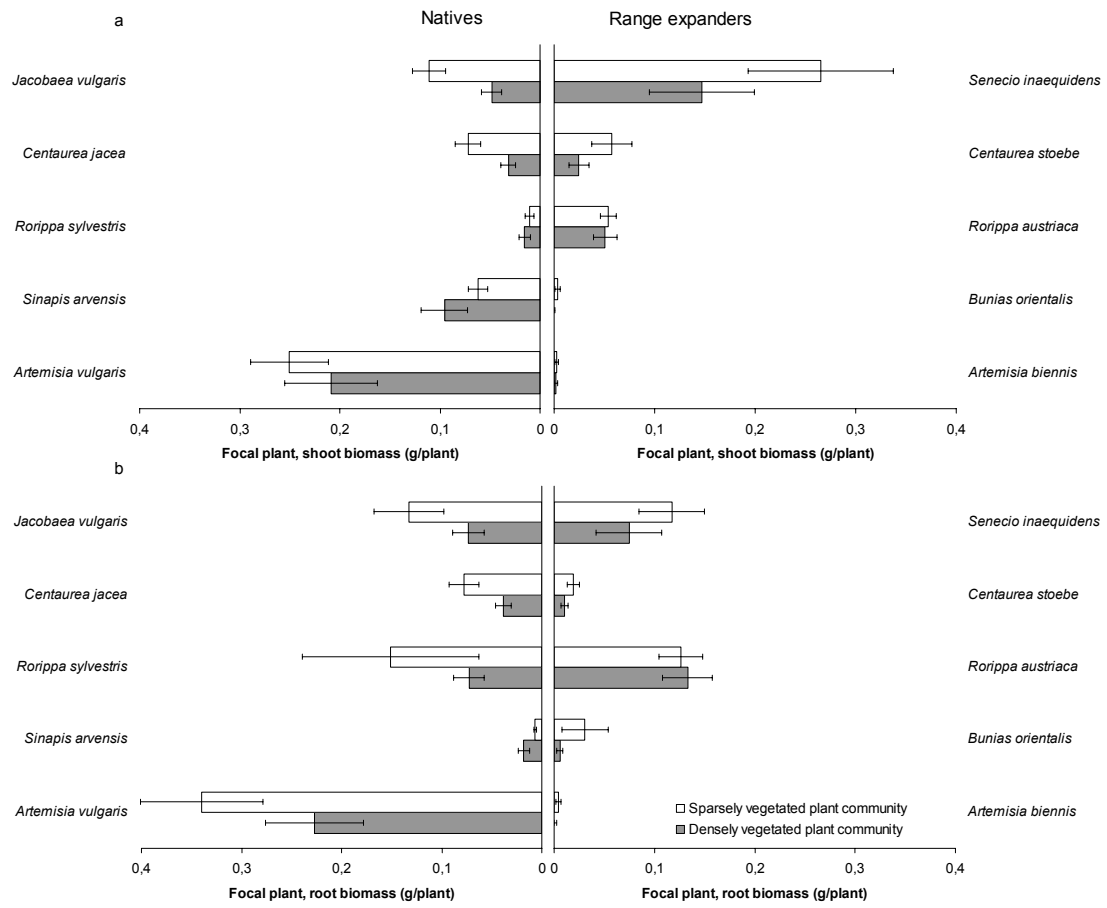
Figure S2 *Field experiment*, mean shoot (panel a) and root (panel b) biomass ± s.e. of the focal plants in pairs. Natives positioned on the left and their range-expanding congeners on the right side in sparsely vegetated and densely vegetated plant communities.

Chapter 7

GENERAL DISCUSSION

The aim of my thesis study was to investigate aboveground and especially belowground interactions of plants that expand their range as a result of climate warming. I tested the hypothesis that range-expanding plant species suffered less from aboveground generalist herbivores and from the soil community than phylogenetically related (congeneric) species that are native in the invaded habitats. I also examined how aboveground and belowground enemy effects would add up in the case of range expanders and congeneric natives. These results will be discussed in the first section. Then I studied the belowground interaction effects with the plants in depth by examining the taxonomic and functional composition of the nematode community, as well as the composition of the soil microbial community in the rhizosphere of range expanding and congeneric native plant species. The results of these studies will be discussed in the second section. In the last section I will discuss whether range-expanding exotic plant species establish better under competition with native vegetation than related native plant species. Here, I tested the hypothesis that range-expanders may benefit from less negative interactions with the soil community than native plant species. I tested this hypothesis both in the greenhouse and in the field. Finally, I will discuss ideas for future research.

Aboveground herbivory and soil feedback effects on range-expanding plants

When plants expand ranges faster than their enemies and when the enemies from the new range do not recognize or feed on the exotic plants, this results in enemy release (Keane & Crawley, 2002; Reinhart *et al.*, 2003). This may provide exotic species with an advantage in interspecific competition with natives, because the latter are still under control of their natural enemies. When enemies or plants in the new range control the invaders by herbivory, pathogenesis or competition this is called biotic resistance (Maron & Vila, 2001). In the first part of my thesis, I examined whether range-expanding plant species may experience enemy release or biotic resistance from the soil community of the invaded range, as well as from polyphagous aboveground invertebrate herbivores.

Plant enemies can be specialists or generalists. Specialist enemies often feed on a limited number of related plant species. Specialists are generally adapted to the defenses of their host plants and can exert strong top-down control of plant productivity and abundance. In the new range, loss of specialists in particular could be beneficial to exotic plants (e.g. Wolfe, 2002), however, the exotics will still be exposed to novel generalist enemies (Joshi & Vrieling, 2005; Müller-Schärer *et al.*, 2004). Generalist herbivores are present in all habitats and colonize new hosts faster than specialists from the new range. In theory, there can be matches, as well as mismatches between plants and their novel herbivores and both have been reported (Parker & Hay, 2005). Therefore, both enemy release and novel plant-enemy interactions can affect exotic plants in new ranges. Whether exotic plants experience reduced enemy impact depends on the net outcome of impacts from the enemies lost from their original range, and the enemies gained in the new range.

In CHAPTER 2 we examined exotic plant exposure to aboveground and belowground enemies using fifteen plant species from a riparian area near the river Waal in the Netherlands. Six of these plants were range-expanding plant species of exotic origin of which three originated from Eurasia (intra-continental range expanders) and three plant species originated from another continent (inter-continental range expanders). We coupled these exotic plants to phylogenetically related natives. Three pairs were coupled to one extra congeneric native plant species to determine whether the observed results may depend on the exotic-native plant species combination. We grew the plants with and without non-coevolved polyphagous (generalist) herbivores, a locust *Schistocerca gregaria* and an aphid *Myzus persicae*. We also exposed all plants to a general soil community from the invaded range and compared their plant-soil feedback responses. We measured plant performance as biomass loss aboveground due to herbivore feeding by the leaf chewer and the sap sucker. These represent the most important insect feeding guilds in the field (Strong *et al.*, 1984). We tested the hypothesis that the plants would not differ in their responses to the polyphagous shoot herbivores, as all plants were equally familiar with them. The fifteen plant species shared no coevolutionary history with *S. gregaria* and *M. persicae.* The aphid had a very cosmopolitan distribution and was highly polyphagous, so that it was unlikely that the selected plants had specific adaptations to this particular aphid species. We also measured the effects of the range-expanders and natives on the performance of these generalist herbivores.

In the same experiment, we determined how all native and exotic plant species developed feedback interactions with the soil community from the invaded range. We hypothesized that both inter- and intracontinental range-expanding plants would develop a less negative plant-soil feedback than the related natives, which would result in less belowground biomass reduction in the range-expanding plants than in the related natives.

Opposite to our hypothesis the generalist herbivores caused significant biomass loss to native plants, whereas their effects on range expanders were much weaker. Intercontinental range expanders were only slightly less negatively affected by herbivory than intracontinental range expanders. The survival of the locust was lower on the range expanders, both inter- and intracontinentals, than on the native species, despite the fact that there was more biomass to consume for the locusts on the range-expanding plants. Aphid numbers were not affected by host plant origin. The negative effects of the range expanders on the locusts could not be explained by C/N-ratio and N-content of the foliage, which are general indicators of food quality for herbivores. However, the levels of phenolic compounds in the foliage of the range-expanders were higher than in the native plant foliage, even when we compared herbivory to the non-herbivore control treatments.

In support of our hypothesis, native plants had more negative feedback interactions than range-expanding plants. The native species had a significant negative plant-soil feedback effect while the range expanders overall experienced a

neutral effect from the soil community. Plant-soil feedback did not differ between plants from inter- and intracontinental origin. It is not new that range-expanding plants can be controlled by negative plant-soil feedback (van Grunsven *et al.*, 2007), although the comparison between inter- and intracontinental range expanders has not yet been made. However, it is remarkable that generalist herbivores feed less on exotic range expanders than on congeneric natives especially because they had the same co-evolutionary history with the native and exotic plant species. Moreover, some of the native plant species used in our experiment are invasive exotics in other continents. This is different from what would be expected from the enemy release hypothesis, since we did not expect to find any kind of release effects from generalist herbivores. In our experiment these effects were most likely caused by the locust, since the locusts are leaf chewers that can be negatively affected by the foliage of range-expanding plants, while the aphids are sap suckers that may not be affected by the plant origin, because they feed on phloem. Apparently, the leaf chewer suffers more from enhanced phenolic contents of the leaves than sap suckers. In addition, also variations in nutrient content, or morphological defenses such as trichomes and leaf toughness, which we did not measure, could have added to this effect (Speight *et al.*, 1999). This might have been caused by the range-expanding process whereby the strongest genotypes of the expanding plant species survives and is able to disperse further north into previous cooler regions while the weaker, less defended genotypes die, and are removed from the gene-pool. So, from our experiment we concluded that range-expanding plant species from a riparian area are somehow better defended against a non-coevolved generalist herbivore than congeneric natives in that habitat.

The Evolution of Increased Competitive Ability (EICA) hypothesis proposes that secondary defenses of exotic plant species can be lowered due to less specialist species attack in the new range (Blossey & Nötzöld, 1995). Therefore, the energy spent in producing secondary defense compounds could be invested in growth, which can lead to further invasion success. Hence, for generalist species constitutive defenses, such as phenolics are always produced at low costs, and are apparently not at all lowered in range-expanding plant species (Joshi & Vrieling, 2005). They have higher levels of this constitutive defense, even when the generalist herbivore is absent. This would mean that range-expanding plants are overall better defended. That enhanced defense might be a temporal effect, which diminish after a few generations after the plant gets established in its new range (Lankau *et al.*, 2009). For range expanders it is to be expected that the release from aboveground specialist herbivores is only a temporal effect and might not even occur for generalist herbivores. Also belowground enemy release is most probably a temporal effect. At some point in time also the slow dispersing soil enemies will catch-up with the range-expanding plant species. However, since we learned from CHAPTER 3 that aboveground and belowground effects can add up linearly, this small temporal release of above- and belowground enemies can give range-expanding exotics a window of opportunity to get established and become abundant in their new range.

Now that we have found this mechanism in a riparian area, it is important to test whether similar processes occur in other habitat types as well.

In CHAPTER 3 I used an expanded version of the data-set of CHAPTER 2 to examine a different set of questions. I tested how individual plants responded to aboveground and belowground plant enemies and then I compared this to their combined effects. As a null hypothesis I assumed that above- and belowground enemy effects would add up in a linear fashion, and that this type of addition does not differ between natives and exotic range expanders. As field experiments have shown different responses of plant community composition to aboveground and belowground plant enemies (Brown & Gange, 1989; Schädler *et al.*, 2004; van Ruijven *et al.*, 2005) it is likely that the strength of aboveground effects do not necessarily correlate with the strength of belowground effects due to defense responses in plants that differ for above- and belowground herbivores or root pathogens. However, since this is difficult to predict, I tested a null hypothesis proposing that for both native and exotic plants the strength of aboveground control by generalist shoot-feeding insects will be indicative of the strength of belowground control by plant-soil feedback.

In line with my hypothesis, combined effects of shoot herbivores and plant-soil feedback on plant biomass are additive both for range-expanding exotic plants and related natives. The results also showed that enemy effects on plant biomass in one subsystem did not influence enemy effects in the other subsystem. In other words, the strength of aboveground defense against herbivore feeders was not indicative of the strength of plant-soil feedback. These results suggest that ultimately plant population control may originate from additive effects of aboveground and belowground enemies. Which of those enemies will provide strongest control may vary among plant species, due to species-specific differences in their sensitivities to shoot herbivory and soil feedback.

Although aboveground and belowground control of plant biomass by generalist shoot herbivores and plant-soil feedback can add up linearly when acting in concert, the strength of negative soil feedback could not be predicted from the responses of the native and exotic range-expanding plant species to the generalist herbivores and vice versa. There are many studies that have proven interactions between above- and belowground biota (e.g. Bardgett & Wardle, 2003, 2010; Bezemer & van Dam, 2005; Soler *et al.*, 2005), but the effect strengths of aboveground and belowground plant control of plant biomass could not be predicted from one another. Moreover, enemy release is only one of the strategies by which invasive exotic plants can become successful in their new habitat. My observation of substantial variation in the strength of the enemy release effect suggests that enemy release should be considered in combination with other mechanisms (Colautti *et al.*, 2004; Levine *et al.*, 2006).

Soil community composition effects on range-expanding plants

In accordance with the enemy release hypothesis (Carpenter & Cappuccino, 2005; DeWalt *et al.*, 2004; Keane & Crawley, 2002; Mitchell & Power, 2003, Williamson, 1996) exotic plant species may lack specific root enemies in the new range. In that case, exotic plants will have less negative soil feedback than related native species (Agrawal *et al.*, 2005). Recent studies has also shown such enemy release effects for range-expanding plant species (van Grunsven *et al.*, 2007). Indeed, comparing soil feedback of a range-expanding plant species between its new and native range suggested that belowground enemies were present in a number of populations of the native, but not in the new range (van Grunsven *et al.*, 2010). On the other hand, some plants repel soil enemies so effectively that they have almost no pathogen accumulation over time (Zhang *et al.*, 2009). In both cases, exotic plants can have an advantage over native plant species that are sensitive to pathogen accumulation, because the type of soil feedback can determine which plants can become dominant and which ones not (Reynolds *et al.*, 2003).

In CHAPTER 4 I used the same fifteen plant species from CHAPTER 2 and CHAPTER 3 from a riparian area near the river Waal to examine how the soil nematode community from the new range responds to exotic plant species compared to related native plants species. Six of the plants from my plant selection were range-expanding plant species of exotic origin. I coupled these six exotic species to nine phylogenetically related native species. I exposed all plant species to a soil community from the invaded range allowing the plants to condition the soil community. Then, the plants were regrown in their own and control soils to test the feedback effects. When most nematode species should have had at least one reproduction cycle the experiment was harvested. I tested the hypothesis that exotic range-expanding plant species have fewer root-feeding nematodes per unit root biomass and that the exotic plant species affect the taxonomic and functional composition of the nematode community different from phylogenetically related native species. In addition, I compared plant origin effects with effects of plant identity. I expected to find the strongest plant origin or plant identity effects in the plant feeders, and diminishing effects with increasing trophic position.

In line with our hypothesis and with the enemy release hypothesis, I found fewer root-feeding nematodes per unit root biomass on range expanders than on related native plant species. This would point at range expanders being resistant rather than tolerant against root-feeding nematodes from the new range. However, lower densities of root-feeding nematodes were partially caused by higher amounts of root biomass in the range-expanding exotic plants compared to the root biomass produced by the related native plant species. But, when we correlated root biomass with the number of root-feeding nematodes we did not found a significant correlation. This would imply that although range-expanding exotic plants had higher root biomass and fewer root-feeding nematodes, root biomass only cannot explain this pattern. Therefore as differences between range expanding and native

plant species were quite consistent across the various types of root-feeding nematodes, our results still suggest that the exotic plants did not promote numbers of root-feeding nematodes as much as related natives. On the other hand, the overall taxonomic and functional nematode community composition was influenced by plant species rather than by plant origin. Indeed, the plant identity effects declined with trophic position, as predicted. This was due to plant feeders being influenced more by species identity than higher trophic level nematodes.

In CHAPTER 5, I examined the soil microbial community composition in the rhizosphere of exotic range-expanding plant species. Soil was collected from the new range and the same six range expanding and nine related natives were planted individually in pots to condition soil microbial communities. After two growth phases I determined the rhizosphere community composition of bacteria and fungi, arbuscular mycorrhizal fungi (AMF) and fusaria. All groups of microbes were analyzed qualitatively and the non-mycorrhizal fungal biomass and fusaria were also analyzed quantitatively. I tested the hypothesis that range-expanding plant species have a different rhizosphere microbial structure than natives.

In line with my hypothesis I found different bacterial rhizosphere communities in native and range-expanding exotic plants. However, these effects did not become significant in the case of fungi, arbuscular mycorrhizal fungi (AMF) and fusaria. Quantitatively, I found less non-mycorrhizal fungal biomass and fewer fusaria genome copies in the rhizosphere of exotic range-expanding plant species than in related native plants. Although this may point at less mycorrhizal dependency and enemy release of the range expanders, there were no differences in fusaria between conditioned and control soil communities, which does not support their role in causing the negative feedback effects to the native plants species. Overall, range-expanding plants appear to have a soil community different from natives, which supports our previous observations that the range expanders had the least negative soil feedback (CHAPTER 2). However, our results did not reveal a causal link between rhizosphere microbial community composition and plant-soil feedback.

Pointing out which rhizosphere biota may play a role in the negative plant-soil feedback effect in our experiments is extremely laborious. To test causality follow-up experiments are necessary in which effects should be tested of a lower density of root-feeding nematodes on plant roots. In case of the microbial community the effect should be tested of adding different microbial suspensions on plant biomass performance. Testing causality with the microbial community is even more constrained since it is not known which genera or species per microbial group are involved in the feedback effect, even not what the functions of the groups are. Groups such as bacteria and non-mycorrhizal fungi may contain saprophytes, symbionts and pathogens, some of them occurring in the rhizosphere but without a function linked to the plant root next to which they occur. With the new generation of sequencing techniques it might be possible to gain further insight in rhizosphere community composition and functioning, however, linking names to function is still

quite constrained in many cases. This keeps the necessity of testing cultured strains of microorganisms onto plant roots to test the function of a certain microbe. This way of testing is very time-consuming and often not possible since only a small part of the bacteria and fungi are culturable. Other techniques, such as functional gene arrays (FGAs) measuring functional genes in the soil with GeoChips are more promising. By studying functional genes in the soil, you directly gain insight in the soil functions of different microbial communities. Even pathogenicity and mutualistic functions of a soil community could potentially be revealed (Gentry *et al.*, 2006; He *et al.*, 2011; Schadt *et al.*, 2005).

Competition of range-expanding plants with a natural background vegetation

Ruderal areas are overall quite disturbed by natural processes, for example flooding or by human causes, such as construction works or soil tillage. Disturbances usually re-set natural succession sometimes by completely opening up sites in the plant community. These open spaces provide opportunities for plants species new for the community, both from native as well as exotic origin, to establish due to temporal low competition with the background vegetation (Burke & Grime, 1996; Hierro *et al.*, 2006). Once the new individuals have become established competion with the background vegetation for light, water and nutrients will start (Dietz & Edwards, 2006; Fargione *et al.*, 2003). Some exotic plant species (Ortega & Pearson, 2005) as well as successful natives are known to be strong competitors in such ruderal areas.

In CHAPTER 6 I compared the early establishment of range-expanding exotics and phylogentically related plant species that are native in the invaded habitats. In a greenhouse I grew five range-expanding plant species and five related natives in sterilized and soil inoculated with non-sterile field soil from the new range, both alone and with a background community of plant species that are also present in the invaded habitat. In the field, I grew the same plants species in artificially created sparse and dense plant communities. I tested the hypothesis that range-expanding exotic plant species establish better under competition with native vegetation than phylogenetically related natives, because range expanders may benefit from less negative interactions with the soil community compared to natives.

Opposite to my hypothesis there was such a strong negative effect of competition from the background community on the shoots and the roots of both range expanding and native plant species when compared to their performance in pots without background community that the shoot and root biomass of the range-expanding plants did not differ from the native congeners, neither alone nor in competition with the background community. Also opposite to what I expected was that soil biota effects were detectable only when the range expanders and natives were grown in monoculture, whereas I had expected that range expanders would benefit indirectly from soil biota when in competition with the background plant community. In the field experiment the shoot and root biomass of range expanding and native focal plants species were also not different. Most focal plants, both range

expanders and natives, appeared to produce more root biomass in sparsely vegetated background communities than in densely vegetated background communities, which is to be expected when plants compete for light. However, this effect was too small to be significant. Since the results of the field experiment are in line with those of the greenhouse, we conclude that under these conditions range-expanding plant species do not benefit from their tolerance, or resistance to soil-borne pathogens from the new range. This stil leaves open the possibility that such benefits may show up on the longer term, when negative plant-soil feedback effects will build-up in the case of natives, but not of range expanders, as demonstrated in CHAPTER 2. However, in order to test that hypothesis, plant-soil feedback studies in the field need to be carried out during a subsequent growing seasons.

Also in the experiments described in CHAPTER 6 there were some species-specific differences in the performance of the plants, which might point at an earlier mentioned statement that most invading plant species have some advantage from enemy release. However, enemy release may not be the only mechanism leading to invasion success (Colautti *et al.*, 2004; Levine *et al.*, 2006) and other factors that influence invasiveness may be species-specific, which can explain why some exotics become invasive and many others not (Williamson & Fitter, 1996). Responses of exotic plant species in monocultures may reveal their invasive potential, but ultimately it depend on all interactions in the field acting in concert which determine whether enemy release or biotic resistance may be the result (Maron & Vila, 2001). Only when sites become very disturbed and vast areas of bare soil are created, the pioneer species under the exotics have opportunities to invade (Burke & Grime, 1996; Hierro *et al.*, 2006). Pioneer species are often not a major concern since they get soon replaced by later successional plant species. However, when the pioneer vegetation is part of a characteristic landscape, for example in drift sands or dune areas, invasion of exotic pioneer species may change biodiversity and disrupt ecosystem functioning.

Ideas for future research

Many studies on exotic plant invasions have focused on the enemy release hypothesis (Keane & Crawley, 2002; Reinhart *et al.*, 2003) as one of the major invasion mechanisms. Most of these studies are based on observations made in the new range (Liu & Stiling, 2006) but in order to know whether species are released from their natural enemies, herbivore pressure from the native range and the new range need to be compared (Hierro *et al.*, 2005). Comparisons of aboveground herbivores and soil pathogens between ranges have been made, but they are either very general in their description of the herbivore groups, or restricted to an individual plant species (Mitchell & Power, 2003; Reinhart *et al.*, 2003 & 2010). Nevertheless, these studies have demonstrated the potential of enemy release as a cause of plant invasions. Climate warming induced range expanders turned out to be less susceptible to herbivores in their new range than congeneric natives. One

question still unanswered is how these range-expanding species are influenced by enemies in their native range, which is an issue for further research. The next step is to study the process of range-expansion itself. Since range-expanders gradually shift range within a continent, they provide a perfect opportunity for detailed studies on changes that may take place along the range-expansion gradient. That approach for example may reveal whether certain enemies are lost or lacking behind the dispersal of the plant species. It would then be very interesting to measure how the biotic interactions work out in plant populations along a transect of expansion. (Van Grunsven *et al.*, 2010) analyzed such a transect, but used a single plant population from outside the transect, which did not allow to study whether range-expanding plants were subject to natural selection.

It would also be interesting to test the levels of plant defence compounds of range expanding plants along the expansion transect. Are well defended species or genotypes better capable of reaching the new range than poorly defended species or genotypes? A similar approach could be taken when studying soil pathogens. First, it needs to be established which pathogens are generally involved in the control process in the native range and then it may be determined whether and how these pathogens act in the new range, as done for an intercontinental invader by (Reinhart *et al.*, 2010). This comparison of measuring release from soil-borne pathogens and the type of root defense (direct or indirect) along a transect from native to new range should be applied to a wider set of plant species in order to detect whether more general patterns exist, or if all these interactions are species-specific.

Since climate change is one of the major global change factors that drive changes in ecosystems (Thomas *et al.*, 2004a; Thuiller *et al.*, 2007; Wall *et al.*, 2010), it is important to be able to make predictions on the future consequences for biodiversity and functioning in natural ecosystems. Currently, the predictions on how plants and their associated communities respond to climate warming are based on climate envelope models. These models predict the distribution of a certain species in terms of their thermal requirements and other mostly abiotic conditions, which all together act like an envelope that matches present climate conditions (Bakkenes *et al.*, 2002; Pearson & Dawson, 2003). Predictions on matches or mismatches are made on the basis of overlapping envelopes. However, species dispersal capabilities are not always incorporated. Barriers in the landscape of unsuitable habitat and habitat fragmentation limits dispersal even further, and are very difficult to incorporate into dispersal models. Most importantly, biotic interactions with other organisms are mostly not considered in these model predictions (Araujo & Luoto, 2007; Brooker *et al.*, 2007; Davis *et al.*, 1998). Therefore, combining models as is advocated by (Araujo & New, 2007) is needed to enhance predictions on how climate change will affect species distributions. These model predictions need to be linked to in depth studies on biotic interactions on a wide set of plant species in a wide range of habitats in order to enhance our knowledge about climate change effects on community composition and ecosystem functioning.

Conclusions

- Range-expanding exotic plant species in riparian ecosystems are better defended against a non-coevolved generalist herbivore than congenerics that are native in the invaded habitats.

- Native plant species suffered more from belowground biotic interactions in their own soil compared to control soil than range-expanding exotic plants.

- Plant population control in both range-expanding exotics and congeneric native plant species may originate from additive effects of aboveground and belowground enemies.

- Temporal release from above- and belowground enemies can provide range-expanding exotics with a window of opportunity to become established and abundant in their new range.

- Exotic range-expanding plant species promoted numbers of root-feeding nematodes less than congeneric natives, however, the overall taxonomic and functional nematode community composition was influenced by plant species rather than by plant origin.

- Range-expanding plants appear to have a soil community that differs from natives, but there was no causal link between rhizosphere microbial community composition and plant-soil feedback.

- Range-expanding exotic plant species did not show a benefit of their resistance or tolerance to soil-borne pathogens compared to congeneric natives when subjected to competition with a background vegetation community. Further studies need to examine if a difference may develop over time, when plant-soil feedback interactions develop.

- In-depth studies on biotic interactions on a wide set of plant species in a wide range of habitats is needed to enhance knowledge about climate change effects on plant community composition and ecosystem functioning.

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SUMMARY

Burning of fossil fuels has raised the level of atmospheric carbon dioxide, which contributes to global climate warming. As a result the mean earth surface temperature has increased faster in the past decades than it has ever done in the previous thousands of years. This rapid climate warming, together with habitat fragmentation, puts a major pressure on many plants and animals. They should either adapt to the warmer climate conditions or disperse in order to keep up with their optimal climatic conditions. The consequence of range expansion is that plants establish new interactions in the invaded ecosystems and that they can be regarded as exotic species. Potential benefits may arise, for example when natural enemies are absent in the new range, which could result in invasiveness of the range shifting species. Although invasive species are a well-studied phenomenon, there is relatively little known about the general mechanisms of biological invasions under climate change.

In this thesis I focus on plant species that expand range due to climate warming. I examined how these range-expanding plants interact with aboveground herbivorous insects and I gave most attention to belowground interactions of range-shifting plant species, congeneric natives and components of the soil food web. I examined how these changed interactions will play a role in the successful establishment of climate change induced range-expanding plants in plant communities that are native inhabitants of the new range.

In the first experiment we examined exotic plant exposure to aboveground and belowground enemies. We used plants that occur in the Netherlands and that originated from Eurasia (intracontinental range expanders) and that originated from other continents (intercontinental range expanders). We compared these exotic plants with phylogenetically related natives. We grew the plants with and without non-coevolved polyphagous (generalist) herbivores, a locust *Schistocerca gregaria* and an aphid *Myzus persicae*. We also exposed all plants to a general soil community from the invaded habitat in the Netherlands and compared their plant-soil feedback responses. The generalist herbivores caused significant biomass loss to native plants, whereas their effects on range expanders were much weaker. Intercontinental range expanders were only slightly less negatively affected by herbivory than intra-continental range expanders. The survival of the locust was lower on the range-expanders, both inter- and intracontinentals, than on the native species, despite the fact that there was more biomass to consume for the locusts on the range-expanding plants. Aphid numbers were not affected by host plant origin. The negative effects of the range expanders on the locusts could not be explained by carbon-nitrogen ratio and nitrogen content of the foliage, which are general indicators of food quality for herbivores. However, the levels of phenolic compounds in the foliage of the range-expanders were higher than in the native plant foliage. Native plants on average had a stronger negative soil feedback interaction than range expanding plants, which

experienced an overall neutral effect from the soil community. The strength of plant-soil feedback interactions did not differ between plants from inter- and intra-continental origin.

Then I tested how plant species responded individually to aboveground and belowground enemies and compared this to their combined effects. I also tested whether the strength of aboveground control by generalist shoot-feeding insects was indicative of the strength of belowground control by plant-soil feedback. Combined effects of shoot herbivores and plant-soil feedback on plant biomass are additive both for range-expanding exotic plants and related natives. The results also showed that enemy effects on plant biomass in the aboveground and belowground subsystem did not influence each other. In other words, the strength of aboveground defense against herbivore feeders was not indicative of the strength of plant-soil feedback. These results suggest that ultimately plant population control may originate from additive effects of aboveground and belowground enemies. Which of those enemies will provide strongest control varies among plant species, due to species-specific differences in their sensitivities to shoot herbivory and soil feedback.

In the next study I examined how the soil nematode community from the new range responds to exotic plant species compared to related native plants species. In line with the enemy release hypothesis, I found fewer root-feeding nematodes per unit root biomass on range expanders than on related native plant species. This would point at range expanders being resistant rather than tolerant against root-feeding nematodes from the new range. However, lower densities of root-feeding nematodes were partially caused by higher amounts of root biomass in the range-expanding exotic plants compared to the root biomass produced by the related native plant species. But when I correlated root biomass with the number of root-feeding nematodes I did not find a significant correlation. This implies that although range-expanding exotic plants had higher root biomass and fewer root-feeding nematodes, root biomass only cannot explain the root-feeding nematode numbers. As differences between range expanding and native plant species were quite consistent across the various types of root-feeding nematodes, our results suggest that the exotic plants did not promote numbers of root-feeding nematodes as much as related natives. On the other hand, the overall taxonomic and functional nematode community composition was influenced by plant species rather than by plant origin. Indeed, the plant identity effects declined in nematodes with higher trophic positions, as predicted. This was due to plant feeders being influenced more by species identity than higher trophic level nematodes, like bacterial and fungal feeders.

As a follow up on this study, I determined the rhizosphere community composition of bacteria, fungi, arbuscular mycorrhizal fungi (AMF) and fusaria. All groups of microbes were analyzed qualitatively and the non-mycorrhizal fungal biomass and fusaria were also analyzed quantitatively. I tested the hypothesis that range-expanding plant species have a different rhizosphere microbial structure than natives. I found different bacterial rhizosphere communities in native and range-

expanding exotic plants, whereas I found no effects in the case of fungi, arbuscular mycorrhizal fungi (AMF) and fusaria. Quantitatively, I found less non-mycorrhizal fungal biomass and fewer fusaria genome copies in the rhizosphere of exotic range-expanding plant species than in related native plants. Overall, range-expanding plants appear to have a soil community different from natives, which supports my previous observations that the range expanders had the least negative soil feedback. However, my results did not reveal a causal link between rhizosphere microbial community composition and plant-soil feedback.

Finally, I compared the early establishment of range-expanding exotics and phylogenetically related plant species that are native in the invaded habitats. In a greenhouse I grew five range-expanding plant species and five related natives in sterilized and non-sterile soils from the new range, both alone and with a background community of plant species that are also present in the invaded habitat. In the field, I grew the same plants species in artificially created sparse and dense plant communities. I tested whether range-expanding exotic plant species establish better under competition with native vegetation than phylogenetically related natives, because exotics may benefit from less negative interactions with the soil community compared to natives. In the greenhouse, there was a strong negative effect of competition from the background community on the shoots and the roots of both range expanders and native plant species when compared to their performance in pots without background community. The shoot and root biomass of the range-expanding plants did not differ from the native congeners, neither alone nor in competition with the background community. Soil biota effects were detectable only when the exotics and natives were grown in monoculture. In the field experiment the shoot and root biomass of range expanders and native focal plants species were also not different. Since the results of the field experiment are in line with those of the greenhouse, I conclude that in this experiment range-expanding plant species do not benefit from their tolerance or resistance to soil-borne pathogens from the new range.

The next step in future research would be to study the process of range-expansion itself. Since range-expanding exotics gradually shift range within a continent, they provide a perfect opportunity for detailed studies on changes that may take place along the range expansion gradient. Since climate change is one of the major global factors that drive changes in ecosystems, it is important to be able to make predictions on the future consequences of range expansions for biodiversity and the functioning of ecosystems.

SAMENVATTING

De verbranding van fossiele grondstoffen hebben de atmosferische koolstof-dioxidewaarde doen stijgen. Dit heeft bijgedragen aan de opwarming van de aarde, waardoor de gemiddelde aardoppervlaktemperatuur in de afgelopen decennia sneller is gestegen dan in de voorgaande duizenden jaren ooit het geval is geweest. Deze snelle opwarming heeft, samen met de versnippering van natuurlijke habitats, een flink negatief effect op veel planten en dieren. Deze organismen moeten zich of aanpassen aan de warmere omstandigheden of migreren om hun optimale klimaatomstandigheden bij te houden. De consequentie van een dergelijke areaal-verandering is dat planten nieuwe interacties aangaan in de gekoloniseerde ecosystemen en als exoten beschouwd kunnen worden. Hierbij kunnen potentiële voordelen ontstaan, bijvoorbeeld als in het nieuwe areaal natuurlijke vijanden ontbreken. Dit kan leiden tot een invasie van de areaaluitbreiders. Invasieve soorten zijn een populair studieobject, maar er is weinig bekend over de algemene mechanis-men die ten grondslag liggen aan invasies door klimaatverandering.

In dit proefschrift beschrijf ik onderzoek naar plantensoorten die door klimaatverandering hun areaal uitbreiden. Ik onderzocht hoe deze areaaluitbreiders interacteren met bovengrondse herbivore insecten en vooral met bodemorganismen. Hierbij werden telkens areaaluitbreiders vergeleken met inheemse planten uit eenzelfde genus. Ik onderzocht hoe interacties met andere organismen een rol kunnen spelen bij succesvolle vestigingen van klimaat-geïnduceerde areaal-uitbreiders in de levensgemeenschappen in het nieuwe areaal.

In het eerste experiment bestudeerden we de blootstelling van plantenexoten aan boven- en ondergrondse vijanden. We gebruikten in Nederland voorkomende planten die afkomstig zijn uit Eurazië (intracontinentale areaaluitbreiders) en die afkomstig zijn van andere continenten (intercontinentale areaaluitbreiders). Deze twee plantengroepen werden vergeleken met inheemse soorten uit dezelfde genera. De planten werden opgekweekt met en zonder ecosysteemvreemde polyfage gene-ralistische herbivoren: de sprinkhaan *Schistocerca gregaria* en de bladluis *Myzus persicae*. Alle planten werden ook blootgesteld aan een levensgemeenschap van bodemorganismen afkomstig van de geïnvadeerde standplaats in Nederland, zodat de plant-bodem-terugkoppeling vergeleken kon worden. De generalistische herbi-voren veroorzaakten een significant biomassaverlies in de inheemse planten, terwijl hun effect op de areaaluitbreiders veel kleiner was. Dit effect was bij de interconti-nentale areaaluitbreiders slechts iets kleiner dan bij de intracontinentale areaal-uitbreiders. De overleving van de sprinkhaan was lager bij beide type areaaluit-breiders dan bij de inheemse soorten, ondanks dat bij deze planten meer biomassa aanwezig was om op te eten. Het aantal bladluizen werd niet beïnvloed door de oorsprong van de plant. De negatieve effecten van de areaaluitbreiders op de sprinkhanen kon niet verklaard worden door twee gangbare indicatoren voor voedselkwaliteit voor herbivoren: de koolstof-stikstof-ratio en het stikstofgehalte van

de bladeren. Het gehalte aan fenolische verbindingen in de bladeren van de areaal-uitbreiders was echter wel hoger dan in die van de inheemse planten. Inheemse soorten hadden gemiddeld een sterker negatieve plant-bodeminteractie dan de areaaluitbreiders, waarbij deze interactie gemiddeld neutraal was. De mate van plant-bodeminteractie verschilde niet tussen intra- en intercontinentale areaaluit-breiders.

Vervolgens toetste ik hoe de plantsoorten reageerden op de losse effecten van boven- en ondergrondse vijanden waarna ik ook het gecombineerde effect bekeek. Ik bestudeerde ook of de grootte van het bovengrondse effect door generalistische bladetende herbivoren indicatief was voor de mate van het ondergrondse effect door plant-bodemterugkoppeling. De gecombineerde effecten van de bladherbivoren en de plant-bodemterugkoppeling zijn additief voor zowel de areaaluitbreidende planten als voor de inheemse planten uit dezelfde genera. De resultaten lieten ook zien dat de effecten van vijanden op de plantbiomassa in het bovengrondse en het ondergrondse compartiment elkaar niet beïnvloeden. Met andere woorden, de mate van bovengrondse verdediging tegen herbivoren was niet indicatief voor de mate van plant-bodemterugkoppeling. Dit betekent dat plantenpopulaties te maken hebben met een additief effect van boven- en ondergrondse vijanden. Welke vijanden het grootste effect hebben varieert van plant tot plant, door soortspecifieke verschillen in hun gevoeligheid voor beide groepen.

In het volgende experiment bestudeerde ik hoe de nematodegemeenschap in de bodem reageerden op plantenexoten in hun nieuwe areaal in vergelijking met de reactie op verwante inheemse planten. Zoals verwacht kon worden uit de 'enemy release' hypothese, vond ik minder worteletende nematoden per eenheid wortelbiomassa bij de exotische areaaluitbreiders dan bij de inheemse planten. Dit wijst erop dat areaaluitbreiders in hun nieuwe areaal resistent zijn tegen deze nematoden in plaats van dat ze ze tolereren. De lagere dichtheden van de worteletende nematoden werden echter gedeeltelijk veroorzaakt door de hogere wortelbiomassa van de areaaluitbreiders in vergelijking met die van de verwante inheemse planten. Wanneer de wortelbiomassa gecorreleerd werd met het aantal worteletende nematoden werden geen significante resultaten gevonden. Dit betekent dat ondanks dat exotische areaaluitbreidende planten een hogere wortelbiomassa en minder worteletende nematoden hadden, de wortelmassa alleen niet de aantallen nematoden kon verklaren. Omdat de verschillen tussen areaaluitbreidende en inheemse planten consequent waren voor de verschillende typen van worteletende nematoden, suggereren de resultaten dat de exotische planten deze nematoden minder bevorderen dan de verwante inheemse planten dat doen. De algehele taxonomische en functionele nematodegemeenschap werd daarentegen vooral bepaald door de plantensoort en niet door de herkomst van de plant (inheems of exotisch). Dit soortsspecifieke effect nam, zoals verwacht, af bij nematoden in hogere trofische niveaus. Dit komt doordat planteneters meer worden beïnvloed door de plantensoort dan nematoden in een hoger trofisch niveau, zoals bijvoorbeeld bacterie- en schimmeleters.

Hierop aansluitend bepaalde ik de levensgemeenschap van bacteriën, algemene schimmels, arbusculaire mycorrhiza schimmels (AMF) en fusariaschimmels rondom plantenwortels (in de rhizosfeer). Alle microbengroepen werden kwalitatief geanalyseerd en de niet-mycorrhiza schimmelbiomassa en fusariaschimmels werden ook kwantitatief geanalyseerd. Ik toetste de hypothese dat areaaluitbreidende exotische plantensoorten een verschillende microbiële samenstelling rondom de wortels hebben dan verwante inheemse planten. Ik vond verschillende bacteriële gemeenschappen tussen beide plantengroepen, terwijl dit voor de algemene schimmels, AMF en fusariaschimmels niet het geval was. Ik vond minder niet-mycorrhiza schimmelbiomassa en minder fusaria genoomkopieën in de rhizosfeer van exoten dan van de inheemse planten. Areaaluitbreidende planten lijken in het algemeen een andere bodemmicrobegemeenschap te hebben dan inheemse planten en dit versterkt de eerdere resultaten waarbij areaaluitbreiders minder negatieve bodem-plantterugkoppeling hadden. Mijn resultaten legde echter niet een causal verband bloot tussen de microbengemeenschap in de rhizosfeer en plant-bodemterugkoppeling.

Ten slotte vergeleek ik de groei van kiemplanten van areaaluitbreidende exotische planten en verwante inheemse soorten in het nieuwe areaal. In een kas liet ik vijf areaaluitbreiders en vijf verwante inheemse soorten kiemen in gesteriliseerde en in niet-gesteriliseerde grond uit het nieuwe areaal. Dit gebeurde in onbegroeide grond (monocultuur van de plant), maar ook in een gemeenschap van plantensoorten die ook in het nieuwe areaal aanwezig zijn (achtergrondgemeenschap). In het veld werd dit experiment herhaald in aangelegde ijle en dichte gemeenschappen. Ik toetste of areaaluitbreiders beter kunnen aanslaan dan inheemse planten als er veel competitie is van inheemse planten, doordat ze mogelijk een minder negatieve plant-bodemterugkoppeling ondervinden. In de kas was er een sterk negatief effect van competitie van de achtergrondgemeenschap op de scheuten en wortels van de areaaluitbreiders en de inheemse planten, in vergelijking met de onbegroeide situatie. De scheut- en wortelbiomassa van de areaaluitbreidende planten verschilde niet van de inheemse planten, noch met noch zonder de achtergrondgemeenschap van planten. Effecten van bodemorganismen waren alleen meetbaar als de exotische en de inheemse planten in een monoculture groeiden. In het veldexperiment konden evenmin verschillen gevonden worden in de scheut- en wortelbiomassa van beide plantengroepen. Omdat de resultaten uit de kas en het veld hetzelfde zijn, concludeer ik dat areaaluitbreidende exoten in dit experiment niet profiteren van hun tolerantie voor of resistentie tegen bodempathogenen in het nieuwe areaal.

De volgende stap in toekomstig onderzoek zou het in kaart brengen van de processen van areaaluitbreiding zelf moeten zijn. Omdat areaaluitbreidende exoten hun leefgebied geleidelijk verplaatsen binnen een continent bieden ze een zeer geschikte mogelijkheid om gedetailleerde studies te doen aan de veranderingen die plaatsvinden langs het traject. Omdat klimaatverandering een van de grootste wereldwijde factoren is die veranderingen in ecosysteem te weeg brengt, is het belangrijk om goede voorspellingen te maken van de effecten van areaaluitbreidende organismen op de biodiversiteit en het functioneren van ecosystemen.

DANKWOORD

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CURRICULUM VITAE

W.E. (Elly) Morriën was born on the 6th of June 1981, in Purmerend, the Netherlands. She completed secondary education (VWO) in 1999 at the Jan van Egmond College in Purmerend. In 1999 she started to study biology at the Free University of Amsterdam. In the same year she commenced the preparation class with viola at the conservatory in Utrecht. In 2003 she received a bachelor degree in biology at the Free University of Amsterdam, for which she performed an internship about heavy metal accumulation in earthworms in floodplain areas. Hereafter, she continued as a master student in ecology, where she modelled the effects of climate change on soil decomposition processes in a pine forest ecosystem. In a second internship she moved to the Metapopulation Research Group at the University of Helsinki, Finland. There she worked for nine months on the connectivity of leaf mining moths on oak trees. In 2005 she received her master degree in ecology at the Free University of Amsterdam. Subsequently Elly worked as a researcher at the Free University of Amsterdam modelling climate induced insect invasions in the Netherlands. In September 2005 she started as a PhD-student at the department of Terrestrial Ecology at the Netherlands Institute of Ecology (NIOO). For more than five years she focused on climate change induced range expanders and their aboveground and belowground interactions with plant mutualists and plant enemies, with this thesis as result.

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PE&RC PhD Education Certificate

With the activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 EC (= 22 weeks of activities)
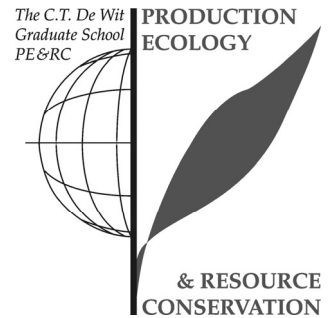
Review of literature (4.5 EC)
- Climate change and invasion by intra-continental range-expanding exotic plants: the role of biotic interactions

Post-graduate courses (5 EC)
- Community ecology, processes, models and applications; PE&RC / SENSE / FE (2005)
- Soil ecology: crossing the frontier between below-and above-ground; PE&RC / SENSE / FE (2007)
- Advanced multivariate analysis of ecological data; University of South Bohemia; Ceske Budejovice (2009)

Invited review of (unpublished) journal (3 EC)
- Annals of Botany: plant invasion ecology (2008)
- OIKOS: plant invasion ecology (2010)
- Biological invasions: plant invasion ecology (2010)

Deficiency, refresh, brush-up courses (3 EC)
- Nematode identification course (2006)

Competence strengthening / skills courses (6 EC)
- Basic course in access; NIOO/Broekhuis Group (2005)
- Presentation skills; WUR (2006)
- Time management; KNAW/FOM (2006)
- Multivariate analysis; PE&RC (2007)
- Introduction to R for statistical analysis; WIAS (2008)
- Database management; NIOO/VLIZ (2008)
- Techniques for writing and presenting scientific papers; Wageningen Graduate Schools (2009)
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PE&RC Annual meetings, seminars and the PE&RC weekend (2.7 EC)
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- PE&RC Day: who is pulling the strings? (2006)
- Workshop plant-insect-interactions (2006)
- NIOO Days (2006, 2007)

Discussion groups / local seminars / other scientific meetings (11.1 EC)
- Terrestrial Ecology (NIOO_KNAW); PhD discussion group (2005-2010)
- Nederlandse entomologendag (2006, 2007)
- Themadag werkgroep exoten; oral presentation 2010 (2006, 2010)
- Netherlands Annual Ecology Meeting oral presentation 2009 and poster presentations 2008 , 2010 (2008-2011)
- Meeting werkgroep bodempathogenen en bodemmicrobiologie van de KNPV oral presentation (2010)
- KNBV: themadag planteninvasies; oral presentation (2010)

International symposia, workshops and conferences (13.6 EC)
- Workshop-Multitrophic Interactions; poster presentation 2008, oral presentation 2010; Goettingen, Germany (2008, 2010)
- 2nd International rhizosphere conference; poster presentation; Montpellier, France (2007)
- BES-Annual meeting; oral presentation; Hertfordshire, United Kingdom (2009)
- GFOE-Annual meeting; oral presentation; Bayreuth, Germany (2009)
- BES-Working group invasive species; oral presentation (first price students presentation); London, UK (2009)
- GFOE-Working group Plant population biology: crossing borders; oral presentation; Nijmegen, the Netherlands (2010)
- NEObiota conference; oral presentation; Copenhagen, Denmark (2010)

Lecturing / supervision of practical 's /tutorials; 2 days (0.6 EC)
- Invited lecture: ecosystem theories on landscape fragmentation; Leiden University (2005, 2006)
- Supervision of 1 HBO student (2006)
- Supervision of 6 MBO students from Laboratorium opleiding Rijnijssel, Arnhem (2005-2010)

Supervision of 1 MSc student; 4 months, 10 days (2 EC)
- Plant soil feedback effects on exotic and native plant species

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