The role of residence time and mutualistic interactions on the strength of plant-soil feedbacks in naturalised *Trifolium.*

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Abstract

Alien plant species may benefit from leaving behind specialised natural enemies when initially introduced to new regions, but the strength of this enemy release may subsequently decline as enemies accumulate, leading to a reduction in the performance of alien plants over time. In addition, alien plants can be dislocated from beneficial interactions with mutualists, limiting their performance.

In this thesis, I examine whether longer-naturalised and more widespread alien plant species experience a weaker escape from soil-borne enemies, as expected if enemy escape is transient. A comparative biogeographic approach was adopted in which plant-soil feedback (PSF) responses were contrasted between the introduced (New Zealand, NZ) and native (Europe) range for 11 *Trifolium* species. A statistical approach was developed to remove the effect of mutualistic nitrogen-fixing bacteria (rhizobia) from PSF responses. The biogeographic difference in PSF between NZ and Europe was then used to quantify the strength of escape from inhibitory PSF for each *Trifolium* species in order to identify whether the strength of escape was correlated with the residence time and geographic spread of the species in NZ. I also identified whether *Trifolium* have retained soil-borne mutualists in NZ by comparing: a) the richness and community structure of arbuscular mycorrhizal fungi (AMF) associated with three *Trifolium* species in NZ and the UK using TRFLP; and b) the symbiotic performance, strain richness and genetic relatedness of rhizobia associated with seven *Trifolium* species in NZ and the UK by conducting a glasshouse experiment, genetic fingerprinting (rep-PCR with ERIC primers) and phylogenetic analysis of the nodD gene.

The strength of biogeographic escape from inhibitory PSF varied considerably among the *Trifolium* species, although most species had similar PSF in both ranges. In contrast to expectations, the strength of escape was not significantly correlated with the residence time of the species. While there was also no overall significant correlation with the geographic spread of the species, less widespread, non-agricultural species experienced a stronger escape from inhibitory PSF, independent of their residence time. The richness and community structure of AMF taxa associated with three *Trifolium* species was similar in NZ and the UK. Rhizobia strains isolated from NZ *Trifolium* had similar nodulation ability as strains isolated from UK plants for all seven *Trifolium* species tested, including agricultural and accidentally introduced species. Genetic fingerprinting indicated that the strain richness of *Trifolium* rhizobia in NZ soils is comparable to that in UK soils. Phylogenetic analysis showed that strains of *Trifolium* rhizobia in NZ are not genetically distinct from UK strains, suggesting that NZ *Trifolium* utilise rhizobia that were co-introduced from Europe.

The *Trifolium* species studied appear to have retained key soil-borne mutualists in NZ that have probably facilitated their successful naturalisation and spread. The findings highlight that the strength of escape from inhibitory PSF is plant species-specific, even among species within a single genus occurring in the same geographic region; this emphasises the need to examine multiple alien species when testing invasion hypotheses. Although the results suggest that the geographic spread and agricultural status of alien plant species may partly influence the strength of escape from inhibitory PSF, a hypothesised decline in the strength of escape with longer residence time of the alien plant species is not supported; the generalisability of this hypothesis is thus questioned.

Keywords: plant-soil feedback, plant-soil interactions, soil biota, belowground mutualisms, mycorrhiza, rhizobia, *Trifolium*, biogeographic, invasion hypotheses, naturalisation, alien, exotic.

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Declaration

Richard Duncan initiated the idea for the statistical approach illustrated in Chapter 2. The glasshouse experiment in Chapter 3 was designed and conducted in partnership with Tasha Shelby, a PhD student at Lincoln University also using the NZ-naturalised *Trifolium* study system. The molecular lab work required to generate the TRFLP data presented in Chapter 4 was conducted by Henk Martens at the Netherlands Institute of Ecology.

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Chapter 1: Introduction

1.1 The enemy release hypothesis

Biological invasions resulting from the introduction of alien species to new regions can cause dramatic ecosystem changes and incur huge financial costs worldwide, the risk of which is growing as species are increasingly moved around the world (Hulme 2009; Pimentel 2011; Jeschke *et al.* 2014). Mitigation of these significant environmental and economic impacts would benefit from an improved understanding of the processes that cause invasion. Explaining why some introduced species fail to establish while others flourish has long been of curiosity to ecologists. Charles Darwin, for instance, originally proposed that species may be more likely to naturalise in communities that lack closely related natives (Darwin's Naturalisation Hypothesis; Darwin 1859; Mack 1996a; Daehler 2001). Since then, a variety of non-mutually exclusive hypotheses have been put forward as potential plant invasion mechanisms (e.g., Mitchell *et al.* 2006).

Alien plant species must pass through a series of stages before becoming invasive, including being transported to a new environment, establishing there, reproducing and finally spreading far from the point of introduction (Blackburn *et al.* 2011). These filters, however, also apply to coevolved organisms that interact with alien plants in their native range (Mitchell *et al.* 2006). This logic forms the basis for one of the most prominent theories used to explain the inordinate success of invaders – the enemy release hypothesis – which states that alien species will lose coevolved natural enemy species such as pathogens, parasites and herbivores that regulate populations in the native range (Darwin 1859; Williams 1954; Elton 1958; Keane & Crawley 2002). The enemy release hypothesis also underlies the principle of biological control of invasive pest species, which attempts to re-establish population control by reuniting pests with a long-lost natural enemy (i.e. bio-control agent) that often originates from the pest's native range (Julien 1987).

The enemy release hypothesis has been tested by two broad types of experimental study: 'biogeographic' and 'within-community' comparisons (*sensu* Colautti *et al.* 2004). Many of the biogeographic studies have shown that plants are attacked by fewer enemy species in their introduced than in their native range (Memmott *et al.* 2000; Mitchell & Power 2003; Torchin & Mitchell 2004) and that alien plant populations can incur less enemy damage (Wolfe 2002; DeWalt, Denslow & Ickes 2004; Hinz & Schwarzlaender 2004; Hawkes 2007; van Kleunen & Fischer 2009). Alien plants have also been shown to escape the most virulent strains of particular pathogens (Reinhart *et al.* 2010) and be impacted less by soil-borne organisms than in their native range (e.g.,

Gundale *et al.* 2014). Plant species that experience greater biogeographic release from enemies have also been shown to be more invasive (Mitchell & Power 2003). These cross-continental biogeographic comparisons can be used to elucidate whether enemy release contributes to differences in the behaviour of plant species between their introduced and native ranges and provide strong tests of the enemy release hypothesis (Hierro, Maron & Callaway 2005).

The second broad type of experimental study used to test enemy release is based on comparisons within invaded plant communities. A number of these within-community comparisons have shown that enemies can have a lower impact on alien plant species relative to closely related natives (congenerics) or other ecologically similar natives (heterospecifics) (Klironomos 2002; Agrawal *et al.* 2005; van der Putten *et al.* 2007). This indicates that alien species may benefit from a competitive advantage over co-occurring natives that allows them to reach higher abundance (Klironomos 2002; Cappuccino & Carpenter 2005). Other within-community comparisons, however, have found that aliens are impacted by enemies to the same degree as natives, counter to enemy release predictions (Blaney & Kotanen 2001; Agrawal & Kotanen 2003; Colautti *et al.* 2004; Dawson *et al.* 2014).

The enemy release effect is generally assumed to be driven more by an escape from specialist coevolved enemies as opposed to generalist enemies (Keane & Crawley 2002; Hinz & Schwarzlaender 2004; Joshi & Vrieling 2005; van der Putten *et al.* 2005; Liu & Stiling 2006; but see Heger & Jeschke 2014). Alien plants may, in fact, still acquire generalist enemies native to the introduced range (e.g., Carroll *et al.* 2005; Gilbert & Parker 2010) that supress their performance (i.e. 'biotic resistance': Maron & Vilà 2001; Levine, Adler & Yelenik 2004; Parker & Hay 2005). Nevertheless, such host-switching of native generalist enemies to feed on alien plants is assumed to be rare, and these generalists are assumed to impose greater impacts on native than on alien plants (Keane & Crawley 2002). Given that closely related plant species are likely to share physiological and chemical traits, they may be attacked by similar enemy assemblages, and thus, aliens with closely related natives in recipient communities may be more likely to acquire enemies (Parker & Gilbert 2004). This may partly explain the lower support for the enemy release hypothesis from within-community studies, as these are often based on phylogenetically-controlled comparisons between alien and native species in the same genus or family (e.g., Agrawal & Kotanen 2003). Such congeneric or confamilial comparisons are therefore considered to be conservative tests of enemy release (Agrawal *et al.* 2005).

While a number of meta-analyses have provided support for key elements of the enemy release hypothesis (Mitchell & Power 2003; Liu & Stiling 2006), the findings of others are equivocal (Colautti *et al.* 2004; Jeschke *et al.* 2012; Heger & Jeschke 2014), or run counter to enemy release predictions

(Maron & Vilà 2001; Levine *et al.* 2004; Chun, van Kleunen & Dawson 2010). This lack of consensus may not be surprising, given that alien plant populations are likely to differ in the degree to which they experience enemy release, varying among the species (e.g., Blaney & Kotanen 2001; Agrawal *et al.* 2005) and region studied (e.g., Reinhart & Callaway 2006). A possible explanation for this context-specificity is that factors such as the residence time of the alien species may influence the strength of enemy release (e.g., Hawkes 2007).

1.2 Dynamic enemy release

While an 'opportunity window' (Johnstone 1986) of enemy release may promote invasiveness when plants are initially introduced to new regions, these benefits may not be permanent. There is evidence that longer-introduced and more geographically widespread alien plant species support a higher richness of enemy species (van Kleunen & Fischer 2009; Mitchell *et al.* 2010). The time since introduction and geographic spread of alien plants have also been identified as good predictors of enemy release strength (Hawkes 2007). The enemy assemblages of alien plants may also become increasingly more specialised with longer time since introduction (Andow & Imura 1994). The findings of these correlative enemy-accumulation studies suggest that enemy release is a dynamic effect that attenuates with the subsequent catch up and accumulation of enemies from the plant host's native range, or as effective enemies are encountered while alien species expand their distribution. Such a transient enemy release has the potential to impose some control on invasive plant populations over time and facilitate the recovery of native plant communities (Flory & Clay 2013). Patterns of enemy accumulation on alien plants are, however, not always consistent (Strong & Levin 1975; Strong, McCoy & Rey 1977), and few studies have tested whether enemy accumulation is associated with a decline in the performance of invasive plants over time (Strayer 2012).

Enemy-accumulation studies have largely been based on the number of enemy species reported on alien plants, but enemy richness may not be a good predictor of the impacts on plant performance. A low number of enemy species can cause significant plant damage, for instance, and the degree to which plant species tolerate enemy damage will vary (Strauss & Agrawal 1999; Chun *et al.* 2010). Studies measuring aboveground enemy pressure and plant performance over invasion chronosequences have had mixed conclusions. Harvey *et al.* (2013) found that the number of invertebrate enemy species hosted by alien populations of *Senecio madagascariensis* was higher at longer-invaded relative to more recently colonised sites in Australia, but plant damage was actually higher at the invasion fronts, contrary to expectations. In line with temporary enemy release, Siemann, Rogers & Dewalt (2006) found that the invader *Triadica sebifera* (syn *Sapium sebiferum*)

experienced higher levels of aboveground herbivory at longer-invaded sites and that this impacted seedling performance.

Furthermore, the degree of regulation that a reunited enemy imposes on an alien plant species may be influenced by evolutionary processes. If relaxed enemy pressure causes rapid evolution of alien plant populations to lose defence traits over time, as predicted by the Evolution of Increased Competitive Ability hypothesis (Blossey & Nötzold 1995), the aliens may be more vulnerable to enemy attack when enemies catch up from the native range (Siemann & Rogers 2003; Siemann *et al.* 2006). Moreover, these enemies may be particularly effective at imposing control if they themselves benefit from enemy escape (Keane & Crawley 2002).

1.3 The influence of soil biota in plant invasion

Until recently, research on plant invasion has been heavily biased towards the effects of conspicuous aboveground enemies such as arthropod herbivores or leaf pathogens. Soil-borne organisms can, however, be key drivers of ecosystem-level processes (Wardle *et al.* 2004), and a growing body of work is now showing that interactions with soil biota can strongly influence the success of alien plant species, either facilitating or inhibiting invasion (Wolfe & Klironomos 2005; Reinhart & Callaway 2006; Inderjit & van der Putten 2010).

1.3.1 Plant-soil feedback

Plant-soil feedback (PSF) describes a process in which plants cause changes to the physical, chemical and biological properties of the soil that, in turn, benefit or inhibit the performance of that plant species and its neighbours (Bever, Westover & Antonovics 1997; Bever *et al.* 2010; van der Putten *et al.* 2013). Such PSF occurs when plants alter the soil environment through processes such as root secretion, nutrient input, changes to soil structure, water-holding capacity or temperature, and the provision of resources or habitats for soil organisms (Ehrenfeld, Ravit & Elgersma 2005). The soil harbours a wide range of micro- and macro-organisms that are antagonistic to plants, including bacterial, viral and fungal pathogens and root-feeding herbivores (Burdon 1987). On the other hand, soil also harbours mutualists that are beneficial to plant growth, the two most significant examples being nitrogen-fixing bacteria and mycorrhizal fungi (Philippot *et al.* 2013; Bever *et al.* 1997 and refs. therein). Controlled PSF experiments can be conducted that quantify the effect of soil biota on plant growth, usually measuring the net effect of all positive and negative interactions with soil biota. Net negative PSF occurs when the overall effect of soil biota reduces plant performance, while positive PSF occurs when soil biota improves plant performance (Bever 2003). The varying strength of net PSF experienced by co-occurring plants can consequently determine the relative abundance of plant

species and mediate vegetation dynamics such as succession (van der Putten, van Dijk & Peters 1993; Bever 2002, 2003; Klironomos 2002; Kardol *et al.* 2007; Kulmatiski *et al.* 2008; Mangan *et al.* 2010; Suding *et al.* 2013; Burns & Brandt 2014).

1.3.2 Escape from inhibitory plant-soil feedback

The PSF experimental approach has been used to investigate the role of soil biota in plant invasion. An increasing number of studies are finding that plant species can experience less suppressive effects from soil biota (less negative PSF) in their introduced relative to their native range (Reinhart *et al.* 2003; Callaway *et al.* 2004, 2011; Reinhart & Callaway 2004; van Grunsven *et al.* 2009; Andonian *et al.* 2012; Yang *et al.* 2013; Gundale *et al.* 2014; Maron *et al.* 2014; but see te Beest *et al.* 2009; Andonian *et al.* 2011; Birnbaum & Leishman 2013). Such escape from negative PSF is likely to enhance the performance of alien plant populations (Reinhart & Callaway 2006) and studies have indicated that this can allow enhanced seedling establishment and increased plant density in the introduced range, thereby facilitating plant invasion (Reinhart *et al.* 2003; Reinhart & Callaway 2004). Alien plant species have also been shown to experience less suppressive PSF than native plants within invaded communities, providing aliens with a competitive advantage, allowing them to attain higher local abundance (MacDougall, Rillig & Klironomos 2011; Klironomos 2002; Niu *et al.* 2007; van der Putten *et al.* 2007; but see Suding *et al.* 2013).

Although the identification of soil-borne agents responsible for net PSF effects can be challenging (Cortois & de Deyn 2011), the most probable cause for less negative PSF in a plant species' introduced range is an escape from inhibitory soil biota, in line with the enemy release hypothesis. A limited number of studies have experimentally separated the effects of different components of the soil community to elucidate their relative contribution to net PSF. Callaway *et al.* (2011) found that alien populations of *Robinia pseudoacacia* encountered less mutualistic arbuscular mycorrhizal fungi than populations in the native range, yet the benefits of escaping inhibitory soil biota appeared to outweigh this disadvantage, as net PSF effects were still more favourable to growth in the introduced range. Other studies have identified that specific soil-borne enemies, such as fungal pathogens (*Pythium* spp.) and specialist nematodes, are absent in the introduced range and likely contribute to the escape from negative PSF (van der Putten *et al.* 2005; Reinhart *et al.* 2010). Nevertheless, an alternative explanation for less inhibitory PSF in the introduced range is that soil-borne mutualists provide superior benefits in introduced-range soils (known as the 'enhanced mutualism hypothesis'; Reinhart & Callaway 2004, 2006), although there is, as yet, little empirical support for this.

The net PSF approach has some significant advantages, despite the difficulties in identifying the agents responsible for plant growth effects. First, the sum of plant growth responses from individual components of the soil community may not equal growth responses from the whole soil community (net PSF), due to synergistic or antagonistic interactions among the components (Bever *et al.* 1997). Mycorrhizae, for instance, can buffer the impacts of plant pathogens (Whipps 2004; Sikes, Cottenie & Klironomos 2009). Second, quantifying the impact of all resident soil biota on plant performance is likely to be the best representation of effects in the field and most useful for understanding the overall role of soil biota in the plant invasion process (Wolfe & Klironomos 2005).

Additional mechanisms by which alien plants can dominate communities include the inhibition of soil-borne mutualisms of neighbouring plant species through allelopathic means (Stinson *et al.* 2006; Vogelsang & Bever 2009; Sanon *et al.* 2009), or the accumulation of soil-borne pathogens that inhibit the growth of natives (Eppinga *et al.* 2006; Mangla & Callaway 2008). Plant invaders can also leave a PSF legacy effect, whereby the soil is left less suitable for the growth of other plant species even after an invader has been removed due to a build-up of suppressive soil biota or inhibitory compounds (reviewed by Eviner & Hawkes 2012).

1.3.3 Dynamic escape from inhibitory PSF

In line with studies showing that enemy richness accumulates with longer introduction and larger geographic spread of alien plant species (Hawkes 2007; van Kleunen & Fischer 2009; Mitchell *et al.* 2010), there is also some evidence that escaping negative PSF may be transient. While few have tested this, Diez *et al.* (2010) found that PSF responses for 12 alien plant species in New Zealand were increasingly negative the longer-introduced and more widespread species were. In addition, Dostál et al. (2013) found that the impact of invasive giant hogweed on native plant communities was lower at longer-invaded sites in Europe and linked this to the occurrence of increasingly negative PSF. Nijjer, Rogers & Siemann (2007) also showed that the invader *Triadica sebifera* (syn *Sapium sebiferum*) accumulated negative PSF in North American soils which limited the recruitment of conspecific seedlings in close proximity to adult trees.

The development of increasingly negative PSF over time in the introduced range likely results from a gradual accumulation of soil-borne enemy densities, species, or more virulent pathogen strains. Indeed, the build-up of suppressive soils in agricultural systems following repeated single-species cropping, historically known as ‘soil sickness’, has been long-realised, and is the basis of crop rotation (Shipton 1977 and refs. therein). The accumulation of species-specific soil-borne enemies is

now known to play a strong role in the development of such negative PSF in agricultural systems (e.g, Olsson & Gerhardson 1992).

The findings of Diez *et al.* (2010), Dostál *et al.* (2013) and Nijjer, Rogers & Siemann (2007) suggest that the accumulation of negative PSF may act as a stabilising process that can reduce the performance and abundance of alien plants over time. This is analogous to successional systems, in which the development of increasingly inhibitory effects from species-specific soil biota can limit the persistence of early successional plant species, promoting their replacement by later successional species (van der Putten *et al.* 1993; Kardol *et al.* 2013). Plant species have also been shown to escape inhibitory soil biota at their range-expanding edges (van Grunsven *et al.* 2007; Engelkes *et al.* 2008), but this may too be a window of temporary benefit. The accumulation of negative PSF may therefore contribute to the boom and bust dynamics of plant invasions or range-shifting plant species (Simberloff & Gibbons 2004; van der Putten 2012), but further research is required to elucidate whether this is a widespread phenomenon.

1.3.4 The role of soil-borne mutualists

While alien plants can profit from escaping enemies, an absence of suitable mutualistic partners in new habitats can limit their establishment and spread (e.g., Seguin *et al.* 2001; Parker, Malek & Parker 2006; Spence, Dickie & Coomes 2011). In some cases, a lack of mutualists may override the benefits provided by the enemy release effect (Morris *et al.* 2007). Most plants rely on some form of mutualism to assist processes such as pollination, seed dispersal or nutrient acquisition. The majority of successful plant invaders, however, appear to have acquired suitable mutualists (Richardson *et al.* 2000a). This can occur when alien plants have access to the same coevolved mutualists present in their native range as a result of the co-introduction of alien plants with non-native mutualists, or the availability of cosmopolitan mutualist taxa (Nuñez & Dickie 2013). Alien plants may alternatively take advantage of non-coevolved generalist mutualists (Nuñez & Dickie 2013) and such novel associations have the potential to produce highly mutualistic (Reinhart & Callaway 2006) or even parasitic effects on the plant.

Mycorrhizal fungi

Mycorrhizal fungi are the most ubiquitous belowground mutualists, associating with 82% of plant species surveyed (Pringle, Wolfe & Vellinga 2011). In general, mycorrhizae enhance the performance of plants by providing nutrients, and in some cases, pathogen and drought protection, in exchange for carbon (Augé 2001; Whipps 2004; Smith & Read 2010). There is evidence that mycorrhizal symbioses are less frequent in alien relative to native floras (Fitter 2005; Pringle *et al.* 2009). This

suggests that reliance on mycorrhizal associations may limit plant invasion and that non-mycorrhizal plants, such as species in the Brassicaceae, may be more likely to successfully establish and invade new regions. The are two principal classes of mycorrhizal association: arbuscular mycorrhizal fungi and ectomycorrhizal fungi (Smith & Read 2010); and their role in determining the success of alien plant species differs (reviewed by Pringle *et al.* 2009).

Ectomycorrhizal fungi (EMF) associate with numerous tree species, often with some degree of host-specificity (Smith & Read 2010) and thus, the success of alien ectomycorrhizal trees can be limited in soils that lack suitable EMF taxa. The EMF genera *Rhizopogon* and *Suillus*, for example, are specific to plants in the Pinaceae family (Dickie *et al.* 2010 and refs. therein) and an absence of compatible EMF in regions of the Southern Hemisphere initially limited the establishment of introduced pine plantations (Richardson, Williams & Hobbs 1994; Nuñez, Horton & Simberloff 2009). This barrier has now been overcome following the human-assisted introduction of suitable EMF to many regions (Vellinga, Wolfe & Pringle 2009; Pringle *et al.* 2009). In fact, now that suitable EMF are available, *Pinus contorta* has become invasive in New Zealand, and its spread is accompanied by introduced EMF (Dickie *et al.* 2010). Australian *Eucalyptus* have also been shown to co-invade with Australian EMF in Spain (Díez 2005). Over 200 EMF species are known to have been introduced to new habitats (Vellinga *et al.* 2009), suggesting that the co-invasion of alien ectomycorrhizal plant hosts with alien EMF may be more common.

In contrast to EMF, compatible arbuscular mycorrhizal fungi (AMF) are assumed to be widely available to alien plants (Richardson *et al.* 2000a). This is largely because many AMF taxa can infect a broad range of host plants (Klironomos 2000) and are globally widespread (Morton & Bentivenga 1994; Öpik *et al.* 2006). While arbuscular mycorrhizal plants are likely to encounter a number of suitable AMF partners in their introduced range, more recent studies have indicated that many AMF taxa have more restricted distributions (Peay, Bidartondo & Arnold 2010; Öpik *et al.* 2010), suggesting that alien plants may be dislocated from a proportion of coevolved AMF partners.

Differences in the taxonomic composition of AMF associated with plants in their introduced and native range may influence the success of alien plants, given that plant growth responses resulting from AMF infection vary greatly depending on the AMF taxa forming the association (Johnson, Graham & Smith 1997; Klironomos 2000; Pringle & Bever 2008). The degree to which AMF protect plants against pathogen attack also depends on the specific combination of plant and fungal taxa (Sikes *et al.* 2009). Communities of AMF are highly diverse (Bever *et al.* 2001) and plants associate with a number of AMF taxa that are connected to many other plant species through a common hyphal network (Newman 1988). Co-occurring plants often receive different levels of benefit from

the AMF network, and this has been shown to mediate competitive interactions among plants and influence the structure of plant communities (van der Heijden *et al.* 1998a; Hartnett & Wilson 2002; Klironomos 2003; Scheublin, van Logtestijn & van der Heijden 2007). Some plant invaders may parasitise the common mycorrhizal network at the expense of other plant species; the invasion of *Centaurea maculosa* into North American grasslands has been facilitated by this mechanism (Zabinski, Quinn & Callaway 2002; Carey, Marler & Callaway 2004). AMF have also been shown to mediate competition between alien and native plant species, the outcome of which depending on the composition of the AMF community (Philip, Posluszny & Klironomos 2001; Bray, Kitajima & Sylvia 2003; Stampe & Daehler 2003; Carey *et al.* 2004; Shah, Reshi & Rashid 2008). Stampe & Daehler (2003), for example, showed that the competitive ability of the invader *Bidens pilosa* relative to native heterospecifics in Hawaii depended on the species of AMF available. The growth benefits that alien plants receive from AMF may therefore depend on the AMF taxa present in recipient soils (Hallett 2006; Inderjit & van der Putten 2010).

Mycorrhizal species richness can also influence the productivity of plant communities, as richer mycorrhizal communities are more efficient at capturing nutrients and can produce more plant biomass (van der Heijden *et al.* 1998b; Jonsson *et al.* 2001). It is thus possible that the richness of compatible AMF available to alien plants, in addition to their taxonomic identities, may influence the success of plant invaders. Dislocation from optimal coevolved AMF, for instance, may mean that alien plants associate with a depauperate number of AMF taxa that provide inferior symbiotic benefits. Nevertheless, despite these possibilities, comparisons of the richness and community structure of AMF taxa associated with plants in their introduced and native range are extremely rare (but see Moora *et al.* 2011).

Nitrogen-fixing bacteria

Leguminous plant species form mutualistic associations with rhizobia bacteria that occupy root nodules and provide plants with the often-limiting nutrient, nitrogen, by converting atmospheric N into bioavailable forms such as ammonium and nitrate (Fisher & Newton 2002). Legumes can perform very poorly in soils that lack suitable rhizobia strains, as has been well documented for agricultural legume crops (e.g., Graham 1981). Despite their reliance on rhizobia associations, many leguminous plant species are among the world's most successful plant invaders (Cronk & Fuller 2001) and the role that access to rhizobia plays in determining the success of invasive legumes is receiving increasing attention (e.g., Birnbaum *et al.* 2012). While many studies have found that legume invaders have been able to form rhizobia associations (Richardson *et al.* 2000a), others have found that the establishment of legumes and their spread away from introduction sites is highly

limited by an absence of suitable rhizobia or low rhizobia densities (Parker 2001; Parker *et al.* 2006; Stanton-Geddes & Anderson 2011; Wandrag *et al.* 2013).

To overcome rhizobia constraints, alien legumes may be accompanied by rhizobia partners from their native range (Stepkowski *et al.* 2005; Wei *et al.* 2009; Rodríguez-Echeverría 2010; Andrus, Andam & Parker 2012; Crisóstomo, Rodríguez-Echeverría & Freitas 2013). The intentional inoculation of legume crop species with coevolved rhizobia, for example, has facilitated the introduction of alien rhizobia to many regions (e.g., Deaker, Roughley & Kennedy 2004). Rhizobia can also be introduced accidentally on the surface of seeds or harboured in other materials (e.g., Pérez-Ramírez *et al.* 1998). The degree to which alien legumes require coevolved rhizobia partners depends on the specificity of the legume-rhizobia association, because the rhizobia relationships of some legumes are generalist, whereas others more specific. *Acacia* species, for example, nodulate with rhizobia in many different genera, such as *Bradyrhizobium*, *Burkholderia* and *Rhizobium*. Such generalist legumes are often capable of forming novel associations with native rhizobia in introduced regions (Pérez-Fernández & Lamont 2003; Ndlovu *et al.* 2013). Other legumes, on the other hand, are more specific in their rhizobia requirements, such as species of *Medicago* that require the rhizobia taxon *Sinorhizobium,* and thus, the establishment of this legume species in new regions depends on the presence of this rhizobia taxon (Béna *et al.* 2005).

Compatible rhizobia strains also vary widely in their nitrogen-fixing abilities and resulting performance benefits provided to legume hosts (Thrall *et al.* 2007; Bever, Broadhurst & Thrall 2013). Rhizobia strains can even parasitise the plant by forming nodules that are ineffective in fixing nitrogen (Denison & Kiers 2004; Pryor *et al.* 2004). Functional differences in rhizobia associations between the native and introduced ranges of legumes are therefore possible, even if alien legumes form root nodules.

1.4 The genus *Trifolium* as a study system

In this thesis, I aim to improve our understanding of how plant-soil interactions influence plant invasion by using a group of European *Trifolium* species introduced to New Zealand (NZ) as a study system. I use a comparative biogeographical approach in which interactions between *Trifolium* species and their associated soil biota are compared between in the introduced (NZ) and native (Europe) range. *Trifolium* is one of the most species-rich genera in the plant family Fabaceae, comprising approximately 255 species, commonly known as clovers (Zohary & Heller 1984; Gillett, Taylor & Collins 2001; Ellison *et al.* 2006). The native distribution of *Trifolium* spans temperate and to a lesser extent sub-tropical regions, with three main centres of diversity: the Mediterranean,

western North America and eastern Africa (Taylor 1985). *Trifolium* are primarily found in grassland habitats and are often particularly abundant in human-managed environments, but some species occur in open woodland, semi-desert and alpine environments (Ellison *et al.* 2006).

Approximately 10% of *Trifolium* species are commercially valuable as important components of agricultural pastureland, most of which are of European origin (Williams & Nichols 2011). Agricultural clover species have been introduced worldwide (Taylor 1985) and have often been accompanied by the inadvertent introduction of other clover species (e.g., Gravuer *et al.* 2008). Many agricultural and non-agricultural clover species have established widespread, self-maintaining populations outside of cultivated areas (USDA, ARS & National Genetic Resources Program). Despite this highly successful naturalisation, introduced clovers are generally not perceived to be problematic species. Some alien clover species, however, potentially meet the criteria to define them as invasive (Richardson *et al.* 2000b; Colautti & MacIsaac 2004), as they can spread significant distances from introduction sites by vectors such as mammals (e.g., rabbit ingestion). *T. repens* and *T. dubium* are in fact included in the 'Global Invasive Species Database' (2014).

The agricultural significance of clover, as for other legume crops, stems from their ability to associate with nitrogen-fixing rhizobia (Graham & Vance 2003). *Trifolium* species are very specific in their rhizobia relationships, nodulating only with the taxon *Rhizobium leguminosarum* biovar *trifolii* (Burton 1985) (Figure 1.1). To establish a clover species in a new location, it was historically common to transfer soil from a field in which that clover species had successfully grown (Burton 1985), and it is now common practise to inoculate agricultural clover seed with commercially-selected rhizobia (Burton 1985). Nevertheless, the presence of clover rhizobia in soil does not necessarily mean that all species of *Trifolium* will be able to successfully establish, as further specificity exists among *Trifolium* species and specific strains of *Rhizobium leguminosarum* biovar *trifolii* (Howieson *et al.* 2005; Melino *et al.* 2012). Rhizobia strains capable of forming root nodules on *T. repens*, for example, may not be cross-compatible with other species such as *T. ambiguum* (Elliot & McIntyre 1998). Compatible rhizobia strains also differ significantly in their nitrogen-fixing ability and resulting growth benefits to *Trifolium* (Howieson *et al.* 2005), meaning that rhizobia present in introduced-range soils may provide inferior or superior symbiotic benefits than rhizobia in the native range.

Trifolium interact with numerous soil-borne antagonists, including many pathogenic fungi, bacteria and viruses, and phytophagous herbivores and nematodes (Barnett & Diachun 1985; Leath 1985; Manglitz 1985; Skipp & Gaynor 1987). *Trifolium* species also form mutualistic associations with arbuscular mycorrhizal fungi that assist nutrient acquisition, particularly phosphorus (Kendall & Stringer 1985). Comprehensive studies examining the effects of inhibitory and mutualistic soil-biota

in both the introduced and native range of plant species' are uncommon. The variety of belowground interactions involving *Trifolium* makes this group of species an interesting study system to investigate how soil biota influence plant invasion.

Figure 1.1. Root nodules on *Trifolium ornithopodioides* formed by *Rhizobium leguminosarum* biovar *trifolii*.

1.4.1 New Zealand-naturalised *Trifolium* study species

There are no native clover species in New Zealand (NZ), but the country now hosts 25 naturalised species, all of which are native to Europe (Gravuer 2004). *Trifolium* species are an integral component of agricultural pastures in NZ (Caradus, Woodfield & Stewart 1996); nine species were introduced intentionally by European settlers in the mid-1800s for this purpose (Gravuer 2004). The remaining 16 species present in NZ appear to have arrived accidentally by seeds 'hitching a ride' as contaminants in imported materials (Gravuer 2004). The research in this thesis focusses on 11 NZ-naturalised *Trifolium* species (Figure 1.2; Table 1.1):

- Four intentional introductions: the perennial species *T. pratense* and *T. repens* that are extensively-used as pasture crops in NZ; the less widely-used perennial *T. fragiferum* (Greenwood 1976); and the non-commercial annual species, *T. dubium* (Boswell *et al.* 2003).

- Seven accidental introductions, all of which are annuals: *T. arvense, T. campestre, T. glomeratum, T. micranthum, T. ornithopodioides, T. striatum* and *T. tomentosum*.

The first recorded dates of naturalisation and current extent of geographic spread of all NZ-naturalised *Trifolium* species were identified by a previous study (Gravuer 2004). The 11 species

used in the present study were selected to span a range of naturalisation dates (1864-1948) and geographic spread in NZ (Table 1.1), in order to test whether longer-naturalised and more widespread species experience a lower strength of release from inhibitory plant-soil feedback. These two variables are only weakly correlated among the species (see Figure 3.1 in Chapter 3), allowing their influence to be examined somewhat independently. This represents a significant advantage of the study system, as previous studies finding evidence for the hypothesis of a transient enemy release have been unable to tease apart the relative contribution of residence time and geographic spread (e.g., Mitchell *et al.* 2010).

We aimed to capture among-species variation in plant-soil feedback responses, despite the close-relatedness of the study species. The species were therefore also selected to span a range of phylogenetic relatedness within the *Trifolium* genus, including species in both subgenera and the four most diverse sections (Table 1.1). *Trifolium* do not have any native congeners in NZ, nor any native closely related species in the 'Vicioid' legume clade (Wojciechowski *et al.* 2000). The study species are therefore more likely to experience a release from specialist enemies than aliens that have native congeners (e.g., Parker & Gilbert 2007). The rhizobia preferences of annual and perennial European *Trifolium* species have been shown to differ (Howieson *et al.* 2005), so species representing both life histories were included in the study.

All 11 study species are naturalised in the Christchurch and Banks Peninsula region on the South Island of NZ, which provided a sampling region comprising a variety of altitudes, habitats and soil types representative of many areas of the country. Distribution records were also available for each species in this region from a floristic survey conducted in the 1980s (Wilson 1992), which assisted the location of naturalised populations. The study species are native to the United Kingdom (UK), apart from *T. tomentosum* which is native further south in Europe (Zohary & Heller 1984). Given that most European settlers in NZ came from the UK, and that a significant amount of trade between the two countries occurred in the late 19th and early 20th centuries (King 2003), the UK is a probable source of introduction of European *Trifolium* species and soil biota to NZ. The UK therefore provided an ideal region from which to sample soil biota representative of the native range of the *Trifolium* species and form a biogeographic comparison with NZ soil biota. Spain was included as an additional sampling region, in order to encompass a wider range of European soil biota and because all 11 *Trifolium* species (including *T. tomentosum*) are native to this region.

T. arvense

T. campestre

T. glomeratum

T. pratense

T. striatum

T. dubium

T. fragiferum

T. micranthum

T. ornithopodioides

T. tomentosum

T. repens

Figure 1.2. The 11 *Trifolium* species used in this study (own images).

Table 1.1. Variables associated with the *Trifolium* species in this study: geographic spread in New Zealand (presence in 30 x 40 km NZ Map Series (NZMS260) grids) and first recorded naturalisation date in NZ according to Gravuer *et al.* (2008); introduction pathway (intentional (I) or unintentional (U) introduction to NZ) according to Gravuer *et al.* (2008); taxonomic group (*sensu* Ellison *et al.* 2006); annual (A) or perennial (P). The final column states which region of the European native range (United Kingdom (UK) or Spain (SP)) soil was sampled from to form a comparison with NZ soil in Chapter 3.

Species	Geographic spread in NZ	Naturalisation date in NZ	Introduction pathway	Annual / perennial	Taxonomic classification	Comparison with NZ
T. arvense L.	83	1880	U	A	subgenus *Trifolium*	UK, SP
T. campestre Schreb.	46	1867	U	A	section *Chronosemium*	UK, SP
T. dubium Sibth.	138	1869	I	A	section *Chronosemium*	SP
T. fragiferum L.	37	1898	I	P	section *Vesicastrum*	UK, SP
T. glomeratum L.	44	1870	U	A	section *Trifoliastrum*	UK, SP
T. micranthum Viv.	23	1854	U	A	section *Chronosemium*	UK
T. ornithopodioides L.	26	1930	U	A	section *Vesicastrum*	UK
T. pratense L.	98	1867	I	P	subgenus *Trifolium*	UK, SP
T. repens L.	181	1864	I	P	section *Trifoliastrum*	UK, SP
T. striatum L.	44	1878	U	A	subgenus *Trifolium*	UK, SP
T. tomentosum L.	21	1948	U	A	section *Vesicastrum*	SP

1.5 Study objectives

While evidence suggests that plant-soil feedback (PSF) may become increasingly negative with longer introduction and larger geographic spread of alien plant species (Diez *et al.* 2010; Dostál *et al.* 2013), the generalisability of this transient escape from inhibitory PSF is unclear. This study tests whether the escape from inhibitory PSF is transient using a biogeographical approach by correlating the strength of biogeographic escape from inhibitory PSF (the difference in PSF between the introduced and native range) with the geographic spread and time since naturalisation of the alien species. Such biogeographical approaches are important in order to rigorously test plant invasion hypotheses (Hierro *et al.* 2005).

A more detailed examination of arbuscular mycorrhizal and rhizobia mutualist communities in the introduced and native range was also required to elucidate whether the study species have retained these soil-borne mutualists in NZ. The degree to which less widespread but successfully naturalised species experience an escape from inhibitory PSF or retain soil-borne mutualists has not been well documented. Negative controls, such as the less geographically successful *Trifolium* species included in this study, are important to assess whether the escape from inhibitory soil biota or the availability of mutualists are major drivers of plant invasiveness (Simons 2003; van Kleunen & Fischer 2009).

The 11 *Trifolium* study species naturalised in NZ were used to answer the following questions:

1. Do *Trifolium* species experience less negative PSF in the introduced (NZ) relative to the native (Europe) range, i.e. have alien populations escaped the effects of inhibitory soil biota?

2. Is the strength of escape from negative PSF weaker for longer-naturalised and more geographically widespread *Trifolium* species in NZ, indicating that the escape from negative PSF is transient?

3. Is the richness of arbuscular mycorrhizal fungi (AMF) associated with *Trifolium* lower in NZ relative to the UK, i.e. have AMF associations been lost in the introduced range?

4. Have annual (non-commercial) *Trifolium* species been dislocated from their optimal rhizobia mutualists in NZ? Is the strain richness of clover rhizobia lower in NZ than in the UK, as expected if a limited number of strains have been introduced to NZ?

1.6 Thesis outline

Chapters 2 - 5 are written as self-contained research papers, and thus, some repetition occurs in the introduction and methods sections. In Chapter 2, a novel approach to the analysis of data from plant-soil feedback experiments is illustrated that uses linear-mixed modelling to overcome some limitations of current approaches. In Chapter 3, I examine whether NZ-naturalised *Trifolium* species escape inhibitory plant-soil feedback relative to their European native range, and whether this escape is transient (addressing questions 1 and 2). In Chapter 4, I examine communities of arbuscular mycorrhizal fungi associated with three clover species in NZ and the UK using the genetic fingerprinting technique TRFLP in order to identify whether the richness of AMF is lower in the introduced relative to the native range (addressing question 3). In Chapter 5, I examine the symbiotic effectiveness, strain richness and genetic relatedness of rhizobia associated with seven *Trifolium* species in NZ and the UK (addressing question 4). Finally, Chapter 6 provides a synthesis of the data chapters, with concluding remarks on the implications of the study and opportunities for further research.

Chapter 2: Unravelling components of plant-soil feedback using a novel mixed modelling approach.

2.1 Abstract

1. Plant-soil feedback (PSF) can arise when plants modify biotic communities in the soil that, in turn, subsequently affects plant growth. A growing body of literature is highlighting that PSF can be an important driver of plant community dynamics and plant invasion. However, current approaches to analysing PSF data have some limitations, including difficulty in handling unbalanced experimental designs and non-independent observations. Furthermore, it would often be helpful to account for the effects of other covariates of plant growth in PSF experiments or quantify plant growth responses resulting from different components of the soil community, but this is not possible using current analysis approaches.

2. Here, we illustrate how to overcome these issues by calculating PSF ratios using a mixed model framework that can handle unbalanced and non-independent data. The uncertainties around mean PSF values produced by this approach are also available for propagation into further statistical operations, which we illustrate by calculating a difference in the strength of mean PSF between two soil provenances of a plant species.

3. Furthermore, covariates known to affect plant growth can be incorporated as fixed effects in the model framework in order to statistically remove their effect from PSF. We show this by modelling the relationship between plant growth and the degree of root nodulation by mutualistic nitrogen-fixing bacteria to estimate the strength of PSF in the absence of this mutualist.

4. This novel approach can be used to calculate PSF from most common experimental designs and offers some significant benefits over standard calculations due to an improved handling of unbalanced data, missing observations, problems with non-independence, and the propagation of uncertainties. The ability to statistically remove the effect of covariates may be useful to account for confounding factors in PSF experiments, such as the degree of pathogen attack. Moreover, the approach can be used to provide insight into the relative contribution of quantifiable components of the soil biota, such as nitrogen-fixing bacteria and mycorrhizal fungi, allowing the effect of these soil components to be statistically teased away from net PSF and partially open up the PSF ‘black box’.

2.2 Introduction

Plants can cause changes to the physical, chemical and biological properties of the soil in which they grow that can, in turn, benefit or inhibit the performance of that plant species and its neighbours: a process known as plant-soil feedback (PSF) (Bever 1994; Bever *et al.* 1997). Negative PSF occurs when the soil modifications reduce plant performance, promoting species turnover and coexistence, while positive PSF increases plant performance, promoting monodominance (Bever *et al.* 1997). An increasing body of work is showing that spatial and temporal vegetation patterns, such as the relative abundance of plant species and plant succession, can be mediated by PSF processes (van der Putten *et al.* 1993; Bever 2002, 2003; Klironomos 2002; Kardol *et al.* 2007; Kulmatiski *et al.* 2008; Mangan *et al.* 2010; Suding *et al.* 2013; Burns & Brandt 2014). The success of many invasive or range-expanding plant species may also be the result of escaping soil-borne enemies that drive negative PSF in the original range (e.g., Callaway *et al.* 2004; Reinhart & Callaway 2004; van Grunsven *et al.* 2010; Maron *et al.* 2014). Despite this extensive literature, many aspects of PSF processes remain poorly understood, including how long alien plant species escape negative PSF, how climate change affects PSF processes and how different components of soil biota contribute to PSF responses (van der Putten 2012; van der Putten *et al.* 2013; Kardol *et al.* 2013).

Controlled PSF experiments can be conducted to quantify the effect of soil biota on plant growth, for instance, by comparing plant performance in soil previously cultivated with a host-specific community of biota, to a control soil lacking that biota. The strength of PSF can be expressed as a feedback ratio that quantifies the performance of plants grown with soil biota relative to the control treatment. These feedback ratios can be calculated in several ways depending on the design and aim of the experiment (reviewed by Brinkman *et al.* 2010), however, current calculation approaches have some limitations, such as in dealing with non-independent and unbalanced data.

In PSF studies, it is common to collect rhizosphere soil from separate field populations of a target plant species and use these soils as replicates in glasshouse experiments in order to encompass spatial variation in the composition of soil biota and resultant PSF effects. However, this sampling design introduces a confounding factor, because the abiotic properties of soil from the different populations will vary and may affect plant growth independently of soil biota. This can be dealt with firstly by using a background soil into which a small inoculation of field soil is made, and secondly, by pairing plants grown in live and control soil treatments, where the soil for each pair was sourced from the same field site (e.g., half of the soil from a site used for a live soil treatment and the remainder sterilised for use in the control). A PSF ratio can then be calculated for each plant pair to quantify the strength of PSF at each site (known as 'pairwise' calculation). Mean PSF and the associated uncertainty can then be calculated from these pairwise ratios, having accounted for

confounding among-site variation in plant growth caused by factors other than soil biota (see Brinkman *et al.* 2010).

These calculations work well when plant growth observations are available for live and control treatments from each sampling site. Unfortunately, it is not unusual for some plants to die during experiments (e.g., van Grunsven *et al.* 2009), causing missing data and unbalanced designs. This is not handled well during the calculation of pairwise feedback ratios, as the loss of one plant in a pair means that data for the surviving plant also has to be omitted, representing a loss of information. In addition, some sampling designs do not have a natural one-to-one pairing of observations. For example, replication could be increased by including multiple replicates of the live and control treatments per site rather than having a single pair per site. It is then not clear how these within-site replicates should be handled because they are not independent. One approach to deal with this non-independence issue is to average plant growth data within the soil treatments from each site, use these to calculate a single feedback ratio for each site, and then average over sites to get an overall mean feedback value (e.g., Maron *et al.* 2014). Invariably, however, the resulting overall uncertainty does not include that associated with averaging data at the site level.

PSF experiments usually measure the net effect of all resident soil biota on plant performance. This net effect will depend on the balance between interactions with biota that affect plant performance positively (e.g., mycorrhizal fungi and nitrogen-fixing bacteria) and negatively (e.g., root-feeders and pathogens). Experimentally isolating different components of soil biota and quantifying their relative contribution to net PSF effects remains a significant challenge (Reinhart & Callaway 2006; Cortois & de Deyn 2011). Nevertheless, the plant growth effects of some soil biota can be quantified. For example, it is often possible to estimate the abundance of mutualistic nitrogen-fixing rhizobia bacteria by assessing the degree of root nodulation on legume hosts, and this is widely observed to correlate positively with legume performance (e.g., Thrall *et al.* 2007; Wandrag *et al.* 2013). Statistically modelling the relationship between the degree of nodulation and plant growth can be used to estimate the growth increment associated with increasing nodulation. This can be used to statistically remove the effect of rhizobia on plant growth by calculating the rate of growth in the absence of nodules, and thus, calculate a PSF ratio that statistically excludes the contribution of this mutualist from other soil biota. A method incorporating this approach into the calculation of feedback ratios would be a step towards opening the PSF effect 'black box', at least in terms of teasing away the influence of quantifiable components of the soil community, such as rhizobia or mycorrhizal mutualists. To our knowledge, though, this approach has not yet been implemented.

Once PSF ratios have been calculated, it may be useful to perform further calculations with these values. For example, we might want to test for significant biogeographic differences in PSF for a

species between its native and introduced range, which would involve testing if the difference between two feedback ratios differed from zero or not. However, it can be challenging to properly incorporate all of the uncertainties involved in the calculation of differences between two feedback ratios.

In this study, we describe a new method for calculating PSF ratios that deals with the issues raised above. Our approach is to embed PSF ratio calculation into a mixed model framework that can accommodate unbalanced study designs and non-independent replicates. The framework can additionally incorporate plant growth covariates in order to statistically remove their effect from net PSF and elucidate the relative contribution of quantifiable components of soil biota. We illustrate the approach by applying it to a small simulated data set, followed by a case study from a PSF experiment using the nitrogen-fixing species *Trifolium glomeratum*. The R scripts used for these examples are provided in text form in Appendix A.

2.3 Methods

2.3.1 PSF calculation in a mixed model framework: simulated data set

We first use a small simulated data set to illustrate how a linear mixed model framework can be used to perform precisely the same calculation as the standard approach to PSF calculation. The simulated data mimicked a typical PSF experiment that would quantify the effect of soil biota on plant performance by growing plants in soil with biota (referred to as live soil) and in a control soil treatment without biota (referred to as sterilised soil) (Table B.1, Appendix B). The simulation consisted of ten growth rate observations from live soil with a mean of 8 and ten observations from sterilised soil with a mean of 5, both including variation randomly drawn from a normal distribution with mean = 0 and standard deviation = 1. The 10 replicates for each soil treatment were handled as if they had originated from different field sites, and thus, there was one live-sterilised plant pair from each site. PSF was quantified using two statistical approaches: the standard approach; and the standard calculation implemented through a linear mixed model.

Standard approach

A variety of different equations have been used to quantify PSF effects, but we used the following PSF ratio because it assists interpretation by producing symmetrical effects sizes in positive and negative values, and does not result in the inflation of type II error (Brinkman *et al.* 2010): $\text{mean}\left(\ln\left(\frac{growth\ rate\ in\ live\ soil}{growth\ rate\ in\ sterilised\ soil}\right)\right)$. For the standard approach, PSF ratios were calculated in a 'pairwise' manner, with each ratio comprised of a live and sterilised plant pair that originated from the same field site, thus controlling for potential variation in abiotic soil properties among field sites. This yielded n = 10 PSF values from the 20 observations (Table B.1, Appendix B). Mean PSF was then

calculated from the 10 pairwise PSF values with 95% confidence intervals calculated by: $t \times (\frac{\sigma}{\sqrt{n}})$; where t is the t-value for 9 degrees of freedom (n-1) at the 0.975 probability level, σ is the standard deviation and n is the number of paired observations.

Model approach (M1)

To repeat this calculation in a model framework, we fitted a linear mixed model (M1) with log-transformed growth rate as the response variable, soil treatment with a constant intercept term as a fixed effect and field site as a random effect. The fixed effect regression coefficients of this model represented the mean difference in growth between live and sterilised soil. As we modelled growth on the log scale, this equated to: $\ln(live) - \ln(sterilised)$; which is mathematically equivalent to the PSF ratio used for the standard calculation: $\ln(\frac{live}{sterilised})$. The soil treatment variable was converted into a binary variable prior to model fitting, where live = 0 and sterilised = 1. This set live soil as the baseline category for the variable so that the difference between live and sterilised soil was expressed the correct way around. The field site random effect replicated the within-site pairwise ratio calculations as performed in the standard approach by allowing the regression intercept and slope estimates to vary for each random effect category (field site). A standard error term for the fixed effect coefficient from the fitted model was used to calculate 95% confidence intervals by multiplying this standard error by the t-value for 9 degrees of freedom at the 0.975 probability level. The data structure, R code and statistical outputs for the models are shown in Tables B.2, B.4 and B.5 in Appendix B. All statistical analyses were implemented in R, version 3.0.1 (R Development Core Team 2013) and models were fitted using restricted maximum likelihood with the lmer function in version 1.7-03 of the R package arm (Gelman & Su 2014). P values for the model parameters were obtained using version 2.0-11 of the R package lmerTest (Kuznetsova, Brockhoff & Christensen 2013).

Error propagation for the model approach

We also used a simulation-based method to capture the uncertainties around mean PSF from the model. Fitted linear mixed models produce a variance-covariance matrix for the fixed effects, which in our model, represented mean PSF, with its associated variance and covariance (Gelman & Hill 2007). We drew 10,000 random values from the normal distribution defined by this variance-covariance matrix using the mvrnorm and vcov functions of version 7.3-31 of the R package MASS (Venables & Ripley 2002). This represented a simulation of values that the PSF ratios may have taken according to the data, in which more probable values were more likely to be drawn. We then calculated confidence intervals around mean PSF at the 95 and 50% levels from the respective quantiles of the 10,000 values. This method of capturing confidence intervals allows uncertainties to be readily propagated into further statistical operations, such as calculating a difference between two mean PSF values (see 'Propagating uncertainties into further calculations' in section 2.3.2).

Advantages of the model approach

A significant advantage of linear mixed models over other statistical methods is their capability to handle unbalanced data, without the need to discard data from groups with missing observations (Littell 2002; Wainwright, Leatherdale & Dubin 2007; Seltman 2012). To deal with this, mixed models implement a method known as 'partial pooling', where model estimates for groups of data (i.e. fixed or random effect categories) that have a lower number of observations and a higher degree of uncertainty are pulled towards the overall group mean (Gelman & Hill 2007). Implementing the calculation of PSF ratios through a linear model framework therefore allows data from PSF experiments that includes missing observations as a result of plants dying to be handled, without the need to omit observations to balance the data.

The partial pooling of data can also account for the non-independence of observations when an appropriate random effect is specified to allow the regression coefficients to vary for each group of non-independent data. This allows within-field site replicates to be handled by the model approach by including a random effect that groups observations from each site. Such within-site replicates then simply provide more information from which the model can estimate PSF at each site, while accounting for the error associated with them.

Calculation of different PSF ratio equations using the model approach (M2)

The model approach described above is useful to perform the PSF calculation stated, however, a simple adjustment to the form of the model provides flexibility to use other PSF equations (some PSF equations are not recommended, however; see Brinkman *et al.* 2010). To illustrate this, we fitted a second model, equivalent to model M1 described above, but with a soil treatment fixed effect specified *without* a constant intercept term (model M2, Table B.4, Appendix B). This model produced fixed effect coefficients that were not expressed as a difference between the fixed effect variable categories as in M1, but instead expressed as estimates of mean growth rates for each fixed effect treatment category, i.e. live and sterilised soil (model output shown in Table B.6, Appendix B), which can then be manipulated to calculate PSF using various equations.

To calculate a different PSF equation, we drew 10,000 random values from the normal distributions defined by the variance-covariance matrix of the fixed effects from M2 using the mvrnorm and vcov functions of R package MASS, version 7.3-31 (Venables & Ripley 2002). This represented 10,000 possible values that growth rates could take in live soil, and 10,000 values that growth rates could take in sterilised soil. These simulated values may then be manipulated to calculate PSF using other equations, and to illustrate this, we chose: $mean(\log(live) - \log(sterilised))$; as used by Klironomos (2002). The data had already been modelled on the log scale, so to achieve this, we calculated $live - sterilised$ over pairs of the 10,000 simulated values. This generated a range of

10,000 possible values for this PSF equation, from which we calculated a mean and captured 95% confidence intervals from the respective quantiles. To compare, we also performed this alternative PSF calculation with the raw data (Table B.1, Appendix B) without using a model. This was achieved by pairing live and sterilised observations from each field site to calculate 10 PSF values, from which we calculated a mean, with 95% confidence intervals based on: $t \times (\frac{\sigma}{\sqrt{n}})$.

2.3.2 PSF calculation in a mixed model framework: case study data set

We also applied the model approach to a case study to demonstrate how the influence of a plant growth covariate can be removed from PSF responses; in this case to remove the effect of nitrogen-fixing bacteria. See Appendix A for R code and Table B.3, Appendix B for data.

Origin of the case study data

The data originated from an experiment that quantified the effect of soil biota on growth of *Trifolium glomeratum* in its introduced (New Zealand, NZ) and native (United Kingdom, UK) range. The experiment was designed to test whether PSF was less negative in NZ relative to the UK, as expected if introduced populations were released from suppressive soil biota.

Glasshouse experiments were conducted in which *T. glomeratum* was grown in live soil and sterilised control soil that originated from five field sites in each country (see Appendix C for full experiment methods). One seed source from NZ and one from the UK were included to address the possibility for post-introduction adaptation of *Trifolium* to soil biota. Being a leguminous species, *Trifolium glomeratum* forms mutualistic associations with nitrogen-fixing root nodule bacteria (rhizobia). Rhizobia can greatly enhance the growth of leguminous plants to the extent that the net effect of the soil community is positive, despite the presence of soil-borne enemies (Bever *et al.* 2013). An absence of suitable rhizobia mutualists may have limited growth of this *Trifolium* species in introduced-range soils, as shown for other legumes (e.g., Parker, Malek & Parker 2006; Wandrag *et al.* 2013). To address this, we included a rhizobia treatment consisting of plants being supplied or not supplied with a commercial rhizobia strain (HiStick® for *Trifolium*, Becker Underwood). There were 80 observations in total: 2 soil provenances (NZ, UK) x 5 field soil collection sites x 2 soil biota treatments (live or sterilised) x 2 seed provenances (NZ, UK) x 2 rhizobia treatments (with or without commercial rhizobia). The data therefore included four 'within-site' replicates of each live and sterilised soil per field site that differed in seed and rhizobia treatment.

Scoring the degree of root nodulation on each plant (see Appendix C for criteria), however, showed that many plants in sterilised soil without rhizobia supplement formed functional nitrogen-fixing nodules (90% in NZ and 10% in Europe). This rhizobia contamination confounded the data, because the degree of root nodulation was a significant positive linear predictor of plant growth rate (linear

regression: slope = 0.003, p = 0.002, R^2 = 0.12, df = 78). We used the model approach to remove the effect of rhizobia from PSF and account for this contamination in sterilised treatments, which had the additional benefit of allowing us to focus on the effects of other soil biota (chiefly soil-borne enemies and mycorrhizal fungi).

Calculation of PSF from the case study data using the model approach (M3 and M4)

We calculated PSF using two linear mixed models: the first quantified net PSF (confounded by rhizobia contamination in sterilised treatments) and the second statistically removed the contribution of rhizobia from net PSF. PSF was quantified for each soil provenance by calculating: $mean(\ln(\frac{growth\ rate\ in\ live\ soil}{growth\ rate\ in\ sterilised\ soil})$. Although the four within-site replicates from each field site differed in seed and rhizobia treatment, for illustrative purposes of this statistical approach, we assumed that they were identical. As a consequence, variation due to differences in seed and rhizobia treatment were treated as residual unexplained variation in the model.

To calculate net PSF, models were fitted with log-transformed growth rate as the response variable, soil treatment (live or sterilised soil) as a fixed effect and field site as a random effect (M3, Table B.4, Appendix B). Separate linear mixed models were fitted for NZ and UK soil as the data originated from independent experiments conducted in different glasshouses. The fixed effect coefficients produced by these two models represented mean PSF for UK and NZ soil, having handled the non-independence of the within-site replicates in a statistically robust manner (see section 2.3.1).

To remove the effect of rhizobia from PSF, we fitted models that werethe same as the two M3 models described above, but also included nodulation score as a fixed effect (M4, Table B.4, Appendix B). These models predicted the linear plant growth response resulting from an increase in the degree of root nodulation. The fixed effect coefficients of these models expressed mean PSF based on the nodulation score x growth rate regression intercept values, i.e. the predicted growth rates of plants with a nodulation score of zero.

The uncertainties around mean PSF from each model were captured from distributions of 10,000 possible PSF values, following the method described for the simulated data set (see section 2.3.1). A breakdown of the M3 and M4 model variables and outputs are shown in Table B.7-B.9 in Appendix B. P values for the model parameters were obtained using version 2.0-11 of the R package lmerTest (Kuznetsova *et al.* 2013).

Propagating uncertainties into further calculations

A benefit of capturing uncertainties around mean PSF values using the simulation-based method (see 'Error propagation for the model approach' in section 2.3.1) is that these uncertainties can be readily propagated into further statistical operations. To illustrate this, we calculated the difference in PSF

between the two soil provenances: PSF in NZ soil minus PSF in UK soil. We drew 10,000 possible values of PSF in NZ and 10,000 in UK soil from the M4 models using the mvrnorm and vcov functions. We then subtracted NZ PSF from UK PSF across rows of these simulated values. A corresponding set of 10,000 possible values for the biogeographic difference were produced, from which we calculated a mean and captured 95% confidence intervals from the 2.5 and 97.5% quantiles of the distribution.

To illustrate how to quantify the contribution of a plant growth covariate to net PSF, we calculated the difference between net PSF (from the M3 models) and PSF with the rhizobia effect removed (from the M4 models). We drew 10,000 possible values of PSF in UK soil and 10,000 possible values of PSF in NZ soil from the M3 and M4 models using the mvrnorm and vcov functions. We then subtracted net PSF values from rhizobia-removed PSF values across rows of the simulated data separately for UK and NZ soil. A corresponding range of 10,000 possible values for the relative contribution of rhizobia were produced for UK and NZ soil, from which we calculated a mean and captured 95% confidence intervals from the 2.5 and 97.5% quantiles of the distribution.

2.4 Results

2.4.1 Simulated data

The standard approach returned a mean PSF value of 0.72 (95% confidence intervals (CI): 0.49, 0.95) and, as expected, identical values were returned by the linear mixed model approach (M1) when CIs were based on the standard error. Capturing uncertainties around the PSF value from the M1 model using the simulation-based method produced slightly different CIs of 0.52 and 0.92. The reason for the difference between the two uncertainty calculations is that the standard error-based method assumes asymptotic normality, which is not approached at the low sample size of this data set; the simulation method is therefore more accurate in this case.

The illustration of calculating PSF with a different equation ($mean(\log(live) - \log(sterilised))$) using the model approach (model M2) produced a value of 0.72 (CI: 0.52, 0.92). The same mean PSF value was produced when this equation was calculated using the standard approach, but with slightly different CI (0.49, 0.95), for the same reasons outlined above.

2.4.2 Case study data

Mean net PSF values for UK and NZ soil produced by model M3 were 0.001 (95% CI: -0.17, 0.18) and 0.220 (95% CI: -0.04, 0.48) respectively (Figure 2.1). The relationship between plant growth rate and root nodulation differed between the two soil provenances, with a lower slope in UK soil than in NZ soil (Figure 2.2). Increasing root nodulation in UK soil therefore provided lower plant growth benefits than in NZ soil, which may have resulted from differences in experimental conditions. Statistically

removing the growth effects of rhizobia using model M4 produced PSF values that were less positive, as expected if removing the effect of a mutualist on plant growth. The degree of shift to less positive PSF differed between the soil provenances: 0.084 and 0.045 lower for NZ and UK soil respectively, in line with the different growth benefits of nodulation in each range (Figure 2.1). The confidence intervals around mean net PSF (M3) and mean rhizobia-removed PSF (M4) were different, but this was expected because the M4 values also included a degree of uncertainty associated with the nodulation x plant growth regressions.

The biogeographic difference in PSF between NZ and the UK from the M4 models was 0.18 (95% CI: -0.09, 0.45), and given that the CI overlap zero, this difference is not significant. The difference between net PSF (M3) and PSF without the rhizobia effect (M4) was 0.05 (95% CI: -0.18, 0.27) and 0.08 (95% CI: -0.26, 0.43) for NZ and UK soil respectively.

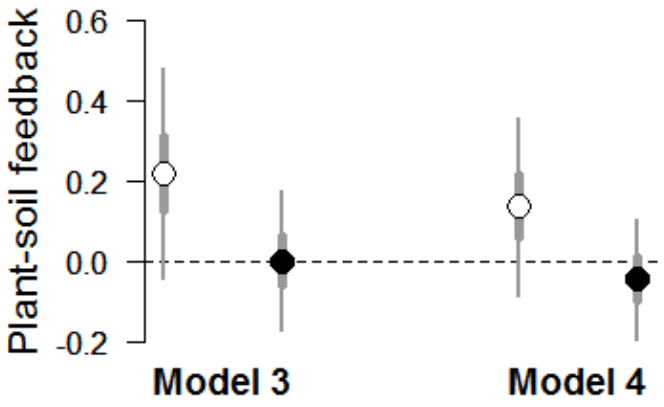

Figure 2.1. Mean plant-soil feedback (PSF) calculated from the case study data set for *Trifolium glomeratum* grown in NZ soil (white points) and UK soil (black points) calculated by two linear mixed model approaches: model M3 quantified net soil biota PSF without removing the effect of rhizobia and model M4 statistically removed the rhizobia effect (see Table B.4, Appendix B). Bars represent 95 (thin bars) and 50% (thick bars) confidence intervals. A PSF value of zero represents no difference in plant growth in live and sterilised soil, negative values represent lower growth in live relative to sterilised soil, and vice versa for positive values.

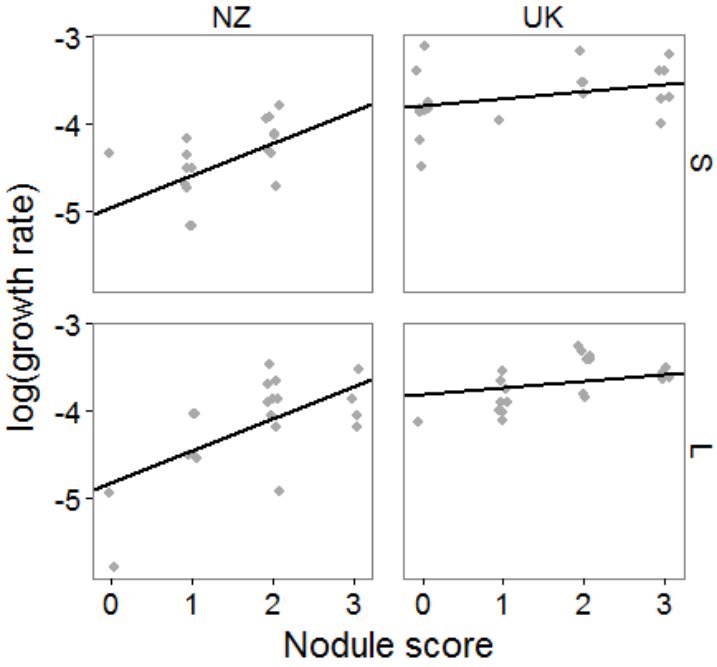

Figure 2.2. Relationships between the degree of nodulation by rhizobia bacteria (nodulation score) and plant growth rate, with linear regressions from the M4 models (see Tables B.4 and B.9, Appendix B). Separate models were fitted for each soil provenance (NZ/UK), in which the intercept terms were allowed to vary by soil treatment (L = live soil biota; S = sterilised soil). Points have been 'jittered' around nodulation scores on the x axis, where a random number has been added to each value to make points more distinct. The intercepts of these regressions indicate the predicted log-transformed growth rate of plants with a nodulation score of zero, which were used to produce estimates of PSF as if rhizobia was absent from the soil.

2.5 Discussion

We introduce a new statistical approach to the calculation of plant-soil feedback (PSF) that can perform precisely the same PSF ratio calculations as the standard approach, but implemented in a linear mixed model framework. It offers some significant benefits over the standard approach, with improved handling of unbalanced and non-independent data. The uncertainties can also be handled using simulation methods so that they can be propagated into subsequent calculations involving PSF values. Furthermore, it offers the novel opportunity to statistically remove the influence of plant growth covariates from PSF and provide some insight into the relative contribution of quantifiable components of soil biota to plant growth.

The ability to include non-independent, within-field site replicates in PSF studies represents a large advantage of the novel approach. Given that plant growth rates would vary among a group of plants grown from a single seed source in an identical soil, it is desirable to include some level of replication at the lowest treatment level. Some PSF experiments that treat field sites as replicates, however, calculate a single PSF ratio from each field site, based on a pair of plants, i.e. one plant in each soil treatment. This is largely because multiple observations for each soil treatment from the same field site are not independent and standard PSF calculations lack a statistically robust way to account for them. The uncertainty around these non-independent observations also needs to be represented in overall mean PSF values, and therefore, averaging such within-site replicates before calculating PSF masks a degree of uncertainty. Our model approach overcomes these issues, as within-site replicates can be handled in a manner that accounts for both their non-independence and associated uncertainties. The inclusion of these within-site replicates then offers significant value in providing more information for the model to estimate PSF from each site. In addition, when data is unbalanced as a result of plants dying, the model approach is able to make use of all available observations, whereas in standard PSF calculations, data from surviving plants would need to be omitted to balance the data.

We also applied the model approach to isolate PSF responses resulting from soil biota other than nitrogen-fixing rhizobia. Doing so accounted for the presence of rhizobia contaminants in sterilised treatments in the case study data set, while also excluding the possibility for different symbiotic benefits of rhizobia between the introduced and native range of the study species. Although the rhizobia-removed PSF response for NZ soil was still positive, representing faster plant growth in live relative to sterilised soil, this may have resulted from the growth benefits of mycorrhizal fungal mutualists in live soil. Using a more continuous variable to assess the abundance of rhizobia, such as the number of nitrogen-fixing root nodules, may also capture better estimations of rhizobia-removed PSF. The approach of removing the effect of plant growth covariates from PSF may also be applied to

other quantifiable soil biota components, such as the ubiquitous belowground mutualist mycorrhizal fungi by including a covariate that quantifies the degree of mycorrhizal colonisation in plant roots (see Vierheilig, Schweiger & Brundrett 2005 for quantification methods).

Furthermore, the actual effect of a covariate on PSF responses can be quantified by calculating the difference in PSF between a model with and without the covariate, i.e. the difference between net PSF and PSF having removed the covariate effect. We illustrated this by quantifying the relative contribution of rhizobia in both NZ and UK soil. Given that rhizobia were present in sterilised treatments in our example data, these estimates are unreliable. However, it does illustrate how the method may be used to statistically disentangle the contribution of different soil biota components. To our knowledge, this is the first description of an approach that can be used to statistically isolate the contribution of different components of net PSF. The ability to isolate such effects statistically may prove very useful, given that it is difficult to achieve this through experimental manipulation. Moreover, retaining an intact soil community in PSF experiments likely represents field effects most accurately, because the sum of effects from experimentally-separated components may not be additive (e.g., Callaway *et al.* 2011), likely owing to interactions among the components. Nodulation by rhizobia bacteria, for instance, can be facilitated by nematodes (Horiuchi *et al.* 2005). Statistically teasing away the effect of quantifiable soil biota components from net PSF may therefore be a preferred method to partially unravel the PSF 'black box', wherever possible.

The removal of covariate effects from PSF may have further applications, for example, investigating how aboveground factors such as the degree of aboveground herbivory influences PSF responses by quantifying and including these factors as covariates. The approach may also be useful to statistically account for other confounding factors, such as infection by unwanted opportunistic aboveground pathogens and pests. The degree of infection by common glasshouse pathogens such as powdery mildew or thrips, for example, may be scored and included as a covariate.

Relationships between response variables and covariates of varying complexity can be accommodated in mixed models. The models that we fitted to remove the rhizobia effect from PSF (M4) assumed that the slopes of the plant growth rate x nodulation score regressions were equal in live and sterilised soil, which was a good description of the data (Figure 2.2). However, if the slope of growth rate x covariate regressions are expected to differ between the soil treatments, a varying-slope, varying-intercept model can be fitted. This could be achieved for our data by specifying an interaction between the soil treatment and covariate fixed effects in the M4 models. Additionally, if the response variable x covariate relationship is non-linear, the approach may be developed using a non-linear mixed model framework (e.g., with R package nlme; Pinheiro *et al.* 2014) or implemented in Bayesian hierarchical models.

Many different PSF experimental designs and ratio equations can be accommodated by the model approach. The soil treatment fixed effect variable in our example models can be easily substituted with a variable comprising the numerator and denominator of the PSF ratio in an experiment. Similarly, the random effect variable included to calculate pairwise PSF ratios can be customised to accommodate other pairwise experimental designs. More complex designs, for which mean PSF needs to be calculated for multiple units such as different plant species or environments can be accommodated in two ways. A single model could be fitted that includes a fixed effect variable referring to both the species/environment unit and the soil treatment, although this will assume a common variance term across the data and may not be appropriate in some circumstances. Alternatively, separate models could be fitted for each unit, as we did for the two soil provenances (NZ and UK) in our case study example.

Chapter 3: Is the escape from inhibitory soil biota transient? A multi-species cross-continental test using naturalised *Trifolium*.

3.1 Abstract

1. Escaping natural enemies is considered to be a key mechanism allowing alien plants to proliferate in new regions, but the degree to which alien plant species benefit appears to be highly variable. Such variation could arise if enemy escape was a transient phenomenon, in which initial escape is followed by a subsequent accumulation of enemies that controls the performance of alien species over time; few, however, have tested this in context of soil biota.

2. We conducted plant-soil feedback (PSF) experiments to quantify the effect of soil biota on the growth of 11 *Trifolium* species in their introduced (New Zealand, NZ) and native (United Kingdom and Spain) ranges. We statistically subtracted the effect of mutualistic rhizobia bacteria from net PSF by modelling relationships between root nodulation and plant growth. The strength of escape from inhibitory PSF was quantified by calculating the between-range difference in PSF for each species. We hypothesised that *Trifolium* experience less negative PSF in NZ than in Europe, but that *Trifolium* species naturalised for longer or more widespread in NZ benefit less.

3. The degree of escape from inhibitory PSF varied considerably among the *Trifolium* species: some benefiting, while others not. The time since naturalisation of the species was not significantly correlated with the strength of escape. While there was also no overall significant correlation between the geographic spread of the species and the strength of escape, more geographically restricted species that were mostly introduced accidentally experienced an average significant escape from inhibitory PSF, whereas more widespread, mostly agricultural, species experienced similar PSF in both ranges. There was also a possible phylogenetic signal, as more closely related species appeared to experience a similar strength of escape.

4. The findings highlight that the strength of escape from inhibitory PSF is species-specific, even among congeners in the same region. This study also shows that less widespread but long-naturalised, non-problematic alien plant species can experience a significant escape from inhibitory PSF, suggesting that the escape from inhibitory PSF may not always be an important determinant of plant invasiveness. Although there was some evidence that the agricultural status, geographic spread and phylogenetic relatedness to other alien species

may influence the strength of escape from inhibitory PSF, a hypothesised transient escape from inhibitory PSF was not supported, and the generalisability of this phenomenon is therefore questioned.

3.2 Introduction

A leading explanation for the success of alien species is that they benefit from escaping natural enemies such as pathogens, parasites and herbivores that regulate populations in the native range (Elton 1958; Keane & Crawley 2002). Evidence for this enemy release hypothesis has firstly been provided by studies within invaded communities showing that alien plants can experience lower enemy impacts than native plants, allowing aliens to attain higher abundance (Klironomos 2002; Agrawal *et al.* 2005; van der Putten *et al.* 2007). Biogeographic comparisons have additionally shown that plants can be attacked by fewer enemy species, less virulent pathogen strains, or incur less damage in their introduced relative to their native range (Memmott *et al.* 2000; Wolfe 2002; Mitchell & Power 2003; DeWalt *et al.* 2004; Hinz & Schwarzlaender 2004; Torchin & Mitchell 2004; Hawkes 2007; van Kleunen & Fischer 2009; Reinhart *et al.* 2010). The evidence from meta-analyses testing the enemy release hypothesis are, however, mixed: some supporting its predictions (Mitchell & Power 2003; Liu & Stiling 2006), others with equivocal findings (Colautti *et al.* 2004; Jeschke *et al.* 2012; Heger & Jeschke 2014), or running counter to its predictions (Maron & Vilà 2001; Levine *et al.* 2004; Chun *et al.* 2010). This lack of consensus may in fact not be surprising, given that the degree to which alien species benefit from enemy release can be influenced by factors such as their residence time (e.g., Hawkes 2007).

While alien plants may benefit from the absence of natural enemies when initially introduced, there is evidence that enemies can subsequently catch up, either through introductions of enemies from the plant host's native range, or as alien plants encounter effective enemies as they spread (Cornell & Hawkins 1993; Andow & Imura 1994; Siemann *et al.* 2006; Flory, Kleczewski & Clay 2011). Meta-analyses have shown that longer-introduced and more geographically widespread alien plant species generally support a higher number of enemy species (Hawkes 2007; van Kleunen & Fischer 2009; Mitchell *et al.* 2010) and experience a lower degree of enemy release (Hawkes 2007). This implies a dynamic enemy release effect in which the strength of release attenuates as enemies accumulate in the introduced range post-establishment. Such a transient enemy release effect may be a fundamental feature of biological invasions that serves to limit the performance of invasive species over time (Flory & Clay 2013). It may additionally underlie the boom and bust dynamics of invasive or range-shifting plant populations (Simberloff & Gibbons 2004; van der Putten 2012).

The overall evidence for a transient enemy release effect, however, remains unclear because the findings of other correlative enemy-accumulation studies are not consistent (Strong & Levin 1975;

Strong *et al.* 1977; Mitchell & Power 2003; Carpenter & Cappuccino 2005). Enemy accumulation patterns can also be influenced by other factors; for example, alien species used for agriculture and forestry may support higher enemy richness, or accumulate enemies more rapidly than non-commercial species (Clay 1995; Mitchell *et al.* 2010; Flory & Clay 2013). Furthermore, the number of enemy species supported by alien plant hosts may not be a good predictor of the control imposed on plant performance (e.g. Harvey *et al.* 2013), given that impacts will vary among enemy species, pathogen strains and plant species (Strauss & Agrawal 1999; Chun *et al.* 2010; Reinhart *et al.* 2010). The enemy-accumulation studies have also been heavily biased towards aboveground enemies, despite a growing body of evidence showing that belowground organisms can play an important role in determining the success of alien plants (Reinhart & Callaway 2006; Inderjit & van der Putten 2010).

The plant-soil feedback (PSF) concept (Bever *et al.* 1997) can be used to quantify the impact of soil biota on plant performance. This commonly measures the net effect of all soil biota, including interactions with antagonistic organisms that inhibit plant growth, and interactions with mutualists that promote plant growth. Negative PSF occurs when soil biota inhibits the subsequent performance of plants, while positive PSF occurs when plant performance is enhanced by soil biota (Bever *et al.* 1997). An increasing number of studies are showing that plant species can experience less negative PSF in their introduced relative to their native range (Reinhart *et al.* 2003; Callaway *et al.* 2004, 2011; Reinhart & Callaway 2004; van Grunsven *et al.* 2009; Andonian *et al.* 2012; Yang *et al.* 2013; Gundale *et al.* 2014; Maron *et al.* 2014; but see te Beest *et al.* 2009; Andonian *et al.* 2011; Birnbaum & Leishman 2013). These findings indicate that the performance of alien plants can be enhanced by encountering soil biota that is more conducive to plant growth in the introduced range. While the acquisition of superior mutualists in the introduced range may explain this trend (Reinhart & Callaway 2006), there is little empirical support for this, and the most plausible explanation is an escape from the impacts of soil-borne enemies in the introduced range. The benefits of this escape from inhibitory PSF may, however, be short-lived, declining as the density and richness of soil-borne enemies accumulates in the recipient soils over time.

Few studies have tested whether the escape from inhibitory PSF is transient, but Diez *et al.* (2010) found that PSF responses for 12 alien plant species in New Zealand were increasingly inhibitory for species resident for longer and with larger geographic spread. In addition, Dostál *et al.* (2013) found that the impacts of giant hogweed (*Heracleum mantegazzianum*) on native European plant communities decreased at longer-invaded sites and linked this decline in plant performance to an accumulation of inhibitory PSF. Rapid accumulation of inhibitory PSF has also been shown to limit the recruitment of seedlings of the invasive plant *Triadica sebifera* (syn: *Sapium sebiferum*) close to adult conspecifics in North America (Nijjer *et al.* 2007). These studies, however, examined PSF within the introduced range, and it is unclear whether these species experience a release from inhibitory PSF

relative to PSF in their native range. There does not yet appear to be a study identifying whether the strength of biogeographic escape from inhibitory PSF (i.e. the difference in PSF between the native and introduced range) is lower for longer-established or more widespread alien plant species, even though such biogeographic comparisons are key to understanding plant invasion mechanisms (Hierro *et al.* 2005).

Here, we test whether the escape from inhibitory PSF is transient by comparing PSF between the introduced (New Zealand, NZ) and native (Europe) range of 11 *Trifolium* (clover) species that span a continuum of naturalisation dates and geographic spread in NZ (Gravuer *et al.* 2008). While the residence time of alien species is often highly correlated with their geographic extent (Pyšek & Jarošík 2005), the naturalisation dates and geographic spread are only weakly correlated among our study species, allowing the relative influence of these two variables to be teased apart. The number of enemies that alien plant hosts encounter and the strength of enemy release experienced can also be influenced by the relatedness of the aliens to native plant species (Mack 1996b; Mitchell *et al.* 2006; Dostal & Paleckova 2011). This can confound comparisons of enemy release among multiple alien species; an issue which we minimise by using multiple species within the same genus that lack native congeners. The study species were nevertheless selected to span a range of phylogenetic relatedness within the genus in order to capture potential variation in PSF responses and examine whether PSF responses were more similar among species in the same taxonomic group.

The performance of legumes such as *Trifolium* can be highly limited in the absence of suitable nitrogen-fixing root nodule bacteria (rhizobia) (Richardson *et al.* 2000a). Rhizobia can even enhance the growth of legumes to the extent that the net effect of the soil community on plant growth is positive, despite the presence of soil-borne enemies (e.g., Bever, Broadhurst & Thrall 2013). Although NZ soil lacks native rhizobia compatible with *Trifolium* species (Greenwood & Pankhurst 1977; Jarvis *et al.* 1977), clover rhizobia (*Rhizobium leguminosarum* biovar *trifolii*) are now widespread in NZ, following their likely inadvertent introduction by early European settlers (Greenwood 1976) and the extensive use of agricultural inoculant for over 60 years (Lowther & Kerr 2011). Nevertheless, we statistically removed the effect of rhizobia from net PSF responses by modelling relationships between plant growth and root nodulation to focus on PSF responses resulting from the remaining soil community (chiefly antagonists and mycorrhizal fungi).

We tested the following hypotheses:

1. *Trifolium* species experience less inhibitory PSF in their introduced (NZ) relative to their native (Europe) range.

2. More widespread or earlier-naturalised *Trifolium* species experience a lower strength of release from inhibitory PSF in NZ.

3. More closely related *Trifolium* species experience a similar strength of release from inhibitory PSF.

3.3 Methods

3.3.1 Study system

We selected 11 NZ-naturalised European *Trifolium* species spanning a range of different naturalisation dates and geographic spread in NZ (Table 1.1 in Chapter 1; Figure 3.1). The species fall into two broad groups based on their geographic spread and agricultural use in NZ: widespread species (present in > 15% of NZ Map Series (NZMS260, 40 x 30 km) grid cells), three out of four of which were intentionally introduced for agriculture; and those with a more restricted distribution (present in < 15% of NZMS grids), only one of seven which was intentionally introduced (Figure 3.1). The selection included species in both subgenera and the four most diverse sections of the *Trifolium* genus (Table 1.1).

Previous studies comparing the impact of aboveground enemies between native and alien *Trifolium* species in California did not support enemy release predictions (Parker & Gilbert 2007; Gilbert & Parker 2010). Studies such as this are, however, likely to be conservative tests of enemy release due to the existence of mutual enemies between native and alien congeners (Agrawal *et al.* 2005). In contrast to California, New Zealand has no native *Trifolium* or closely related species in the 'Vicioid' legume clade (Wojciechowski *et al.* 2000); NZ-naturalised *Trifolium* may therefore be more likely to escape enemies (e.g., Strauss, Webb & Salamin 2006).

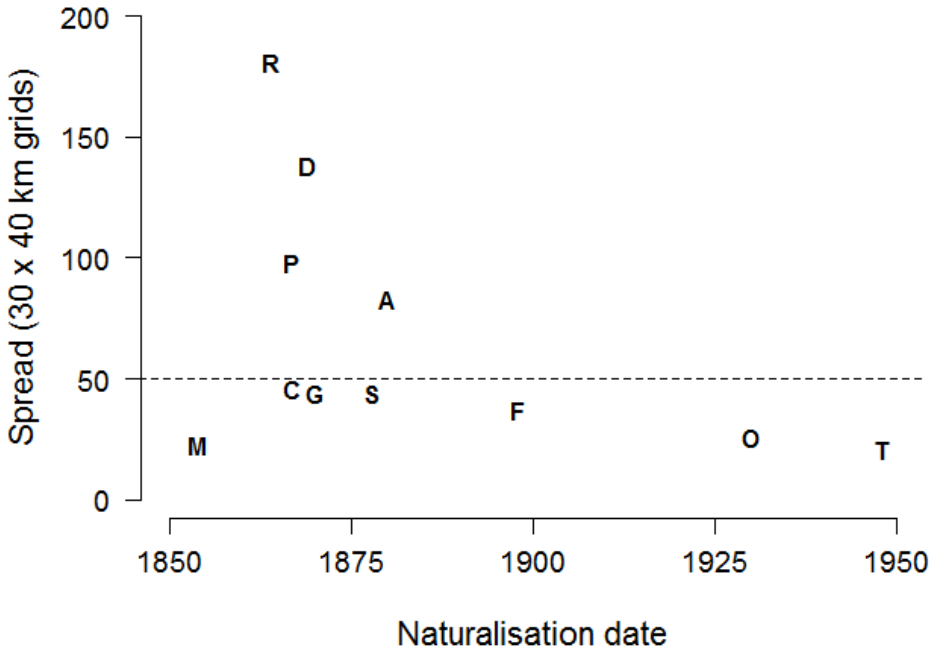

Figure 3.1. Naturalisation dates and geographic spread (presence in 30 x 40 km New Zealand Map Service (NZMS60) grids squares) of *Trifolium* study species in New Zealand (data from Gravuer *et al.* 2008). Letters indicate species: *T. arvense, T. campestre, T. dubium, T. fragiferum, T. glomeratum, T. micranthum, T. pratense, T. repens, T. striatum, T. ornithopodioides* and *T. tomentosum*. Pearson's correlation coefficient = -0.46; p = 0.15. The dashed line represents the spread value at which we split species into two groups: less widespread, mostly accidentally introduced species (below the line) and widespread, mostly agricultural species (above the line). Species D, F, P and R were introduced to NZ intentionally for agriculture.

3.3.2 Experiment overview

We quantified plant-soil feedback (PSF) responses for each *Trifolium* species in the introduced (NZ) and native (Europe) range by calculating PSF ratios that compared plant growth rate in soil with biota (inoculated with live field soil) relative to soil lacking that biota (inoculated with sterilised field soil). Separate glasshouse experiments were conducted within each range as it was not possible to transfer soil to a single location due to the costs incurred, quarantine regulations in each host country and because soil biota may not have survived temperature extremes experienced during transportation. Quantification of relative PSF ratios within a common environment in each range subsequently allowed us to make between-range comparisons, having accounted for differences in glasshouse conditions. We statistically removed the effect of rhizobia from net PSF responses and then quantified the strength of biogeographic release from inhibitory PSF for each *Trifolium* species by calculating the difference in PSF between the introduced and native range.

3.3.3 Soil collection

In New Zealand, soil was collected from Banks Peninsula and Christchurch, as this region provided a range of soil types and environments broadly representative of the habitats that *Trifolium* occupy in the South Island of NZ. Distribution records from a floristic survey conducted in the 1980s (Wilson 1992) assisted in the location of naturalised populations of each *Trifolium* species. In the native European range, soil was collected from two regions: the south coasts of England and Wales in the United Kingdom (UK); and the Basque Country to Catalonia in Northern Spain. The UK was chosen because this country is a likely source of European soil biota introduced to NZ, given the extent of trade and exchange of biological materials between the two countries (Allen & Lee 2006; Walrond 2012). Botanical records from the Botanical Society of Britain and Ireland helped to locate British populations. Northern Spain was chosen in addition to the UK in order to encompass a wider variety of native-range soil biota. Three botanical databases were used to locate Spanish populations: Aranzadi (available at: http://www.aranzadi.eus/botanica/herbario); Anthos (available at: http://www.anthos.es/); and records from the Jaca Herbarium, Instituto Pirenaico de Ecologia, Jaca, Spain. Nine of the 11 *Trifolium* species were sampled in Spain, nine in the UK (seven of which from both native regions), and all 11 in NZ.

Five naturalised populations of each species per geographic region were selected encompassing sites with a variety of abiotic conditions (soil pH, soil texture and habitat) and a possible corresponding variety of soil communities (see Appendix D for population locations). To provide a representative sample of soil biota from each population, rhizosphere soil was collected from below 10 randomly-selected plants at least one metre apart. These field soils were air-dried if wet, sieved to 4mm, visible macrobiota (i.e. grubs) removed and then homogenised from each population to form five independent replicates of soil communities from each region per species. Soil sampling equipment was sterilised using 10% household bleach or 10% Dettol to prevent soil biota being transferred between replicates. Before the experiment setup, soil was stored at 4° C during the 2012 season in NZ. There was no access to a fridge during European fieldwork, so soil was stored at room temperature for the remaining seasons.

3.3.4 Glasshouse experiments

Glasshouse experiments with NZ soil were conducted at Lincoln University in NZ, while experiments with European soil were conducted at the Netherlands Institute of Ecology (NIOO-KNAW), Wageningen. Two separate rounds of experiment were run at each location during two successive years (2012 and 2013) using soil collected the same year because there were constraints on seed supply for some species. One half of the homogenised field soil from each *Trifolium* population was sterilised. Live or sterilised field soil was used to inoculate a homogeneous, sterilised 'background'

soil in each glasshouse. The field soil inoculant formed only 10% of the total soil volume in each plant pot (total pot volume of 1.3 L in NZ 2012, otherwise 1 L), thereby diluting the effect of any abiotic differences among the field soil inoculants. Soil sterilisation was achieved by gamma irradiation in Europe (> 25 KGray, Ede Isotron, The Netherlands). Gamma irradiation was not feasible in NZ, so soil sterilisation was conducted by two rounds of autoclaving on a cycle held at 121° C for 20 min, with a minimum of 48 hrs between runs. Autoclaving soil did not induce chemical changes that would be damaging to plant growth and organic matter and total nitrogen content of background soils were comparable between the two glasshouses (see Appendix E).

We included a rhizobia treatment in the 2012 experiments, consisting of supplement or no supplement with a commercial *Rhizobium leguminosarum* biovar *trifolii* strain (HiStick® for *Trifolium*, Becker Underwood, strain RGAAE19). The original aim of this treatment was to identify whether optimal rhizobia strains were lacking in NZ soils, however, production of this rhizobia preparation ceased, so this treatment had to be dropped from 2013 experiments. In lieu of this in 2013, we included two pots of live or sterilised soil from each field site. The number of observations for each species within each region varied between 16-40, depending on the number of plants that died after transplant and because some species were grown in both 2012 and 2013 within each glasshouse, using soil collected the same year (Appendix F).

Seed was sourced from native populations of each species in the UK or Spain (see Appendix G for seed source details). Seed from the UK was grown in UK and NZ soil, and seed from Spain was grown in Spanish and NZ soil. For each comparison, the same seed sources were used in each glasshouse to ensure between-range comparability. Seeds were surface-sterilised with a 2 min immersion in 10% sodium hypochlorite (household bleach) with a drop of Tween20 surfactant and rinsed thoroughly with de-ionised water. Hand scarification was performed with a scalpel and seeds were stratified in germination paper or in seed trays at 4° C for 24 h to break dormancy. Seedlings were germinated in either sterilised sand or sterilised glass beads in an incubator with species-specific conditions: between 12 - 18° C with 8 - 12 h light per day. Similar-sized seedlings were transplanted into pots inoculated with live or sterilised field soil and the soil surface of each pot was covered with aluminium foil to reduce evaporation and supress weed growth. Seedlings that died within the first week were replaced. Pots were supplied with adequate water in NZ 2012, but to improve standardisation, pots were watered to an equal weight weekly for all remaining seasons. Outbreaks of opportunistic thrips were controlled by releasing biocontrol mites (*Amblyseius cucumeris*; twice in NZ in 2013 and once in Europe in 2013). A non-systemic fungicide (Chlorotek) was applied directly to all leaves of *Trifolium campestre* and *T. dubium* plants in NZ in 2012 to control opportunistic powdery mildew infection. Plants of the same species grew at different rates between the two glasshouses, so

the formation of flower buds on any plants was used as a cue to harvest all plants of that species. Roots and shoots were separated, oven dried at 70° C for at least 48 h and then weighed.

To evaluate the degree of effective nitrogen-fixation by rhizobia, root nodulation of each plant was scored at the time of harvest on a 0-3 scale adapted from Thrall et al. (2007) and Corbin et al. (1977), based on the size, number, position and colour of nodules. Functional nitrogen-fixing nodules are pink due to presence of the pigmented protein leghaemoglobin, whereas ineffective nodules are white (Somasegaran & Hoben 1994). Nodule position on the root system indicated the plant growth-stage at which rhizobia infection occurred. A score of zero represented no nodulation or white (ineffective) nodules only. Scores of 1-3 represented increasing degrees of effective N-fixation: 1 for a low number of small (< 1mm) pink nodules predominantly at lower parts of root; 2 for an intermediate number of larger pink nodules (many >1mm), some present at the root crown (top 2cm of the root system); 3 for abundant large (many > 1mm) pink nodules, particularly at the root crown.

3.3.5 Statistical analyses

Plant growth rates were calculated by dividing total dry-weight biomass by the number of glasshouse-grown days. Relationships between the degree of rhizobia root nodulation and plant growth rate were examined prior to the quantification of plant soil feedback (PSF) (see Appendix H). Unfortunately, rhizobia contaminants were present in many pots of sterilised soil without commercial rhizobia supplement (see Appendix H). This contamination confounded PSF quantification because the degree of root nodulation was a significant linear predictor of plant growth rate (linear regression: $p = < 0.001$, $R^2 = 0.22$, $df = 897$). To account for this, we statistically modelled the relationship between plant growth and nodulation and used the intercepts of these regressions (estimates of plant growth in the absence of rhizobia) to calculate PSF as if there were no rhizobia in the soil. Removal of the rhizobia effect also excluded the possibility for differences in the symbiotic benefits of rhizobia between the introduced and native range, allowing us to focus on PSF effects driven by other soil biota.

Removal of the rhizobia effect was achieved by fitting a linear mixed model in which log-transformed growth rate was specified as the response variable, with three fixed effects: 1) a categorical ‘group’ variable that referred to soil treatment (live or sterilised), species, seed origin (UK/Spain) and soil origin (NZ/UK/Spain); 2) nodulation score; and 3) rhizobia treatment. Live soil was set as the baseline category for the soil treatment variable and the categorical ‘group’ fixed effect was specified without a constant intercept term. Two categorical random effect variables were included that: 1) allowed the intercepts of the growth rate x nodulation regressions to vary by live and sterilised soil for each species, seed and soil origin; and 2) allowed the regression intercepts to vary by rhizobia treatment, year of experiment and field soil collection sites for each species, seed and soil origin. The fixed effect

intercept coefficients from the fitted model provided statistical estimates of growth rates for each species, seed and soil origin in live and sterilised soil according to the intercepts of the regressions (i.e. a nodulation score of zero and no commercial rhizobia addition, representing plants without access to rhizobia). See Appendix I for further explanation of the model variables and model output statistics. Statistical analyses were performed in R version 3.1.0 (R Development Core Team 2013) and linear mixed models were fitted using restricted maximum likelihood with the lmer function in version 1.7-03 of the R package arm (Gelman & Su 2014).

We drew 10,000 random values from the posterior distributions of the modelled fixed effects, as characterised by the mean, variance and covariance of the fixed effect coefficients, using the sim function of the R package arm (Gelman & Su 2014). These simulations represented possible values of plant growth rates, in which more probable values were more likely to appear in the simulation (Gelman & Hill 2007), consisting of 10,000 values for live soil and 10,000 for sterilised soil, for each species, seed and soil origin. These values were used to calculate 10,000 possible PSF values for each species, seed and soil origin by subtracting the growth rates in live soil from those in sterilised soil over rows of the estimated values. We calculated mean PSF responses from the 10,000 possible PSF values for each species, seed and soil origin, with 95 and 50% confidence intervals according to the respective quantiles of the values (see Figure J.1 in Appendix J). To verify that this statistical approach was robust, we compared the resulting PSF responses to those calculated: a) without model estimation (the standard approach); and b) using model estimation but without removing the rhizobia effect (see Appendix I).

Biogeographic differences in PSF were used to quantify the strength of escape from inhibitory PSF for each species by subtracting PSF in NZ soil from PSF in European soil, using three biogeographic comparisons: NZ - UK; NZ - Spain; and NZ - Europe overall. For each comparison per species, 10,000 possible biogeographic differences were calculated by subtracting the 10,000 estimates of PSF in NZ soil from 10,000 estimates of PSF in European soil. We then calculated mean biogeographic differences from the 10,000 values for each comparison per species and captured 95 and 50% confidence intervals from the respective quantiles around the values. A biogeographic difference of zero represents no difference in PSF between the ranges; a positive biogeographic difference indicates that NZ soil biota had a less inhibitory effect on plant growth than European soil biota, suggesting an escape from inhibitory PSF; negative values indicate that NZ soil biota inhibited plant growth more than European soil biota. The significance of each biogeographic difference was assessed by whether 95% confidence intervals overlapped zero.

We used a Bayesian framework to estimate mean biogeographic differences in PSF for groups of species: a) over all species for each biogeographic comparison (NZ - UK; NZ - Spain; NZ - Europe) to

test for general trends for an escape from inhibitory PSF; b) over two groups of species according to their geographic spread and agricultural use in NZ (see Figure 3.1); c) over species within taxonomic groups (see Table 1.1). The fitted Bayesian regression models were based on Duncan *et al.* (2011), in which regression coefficients for each group of species were modelled as random effects drawn from a common distribution of a particular species mean. By taking into account the variance around each species' mean biogeographic difference, the models placed more weight on means with more precise regression coefficient estimates. Species with means that showed higher variation thereby had a lower influence on the species group mean. Non-informative prior distributions were specified and each model was run for 20,000 iterations with a burn-in of 10,000 iterations. This Bayesian framework was also used to test for an overall linear trend between the biogeographic differences in PSF and the time since naturalisation or geographic spread of the species in NZ. Bayesian regression models were implemented with the JAGS software (Plummer 2003) via version 0.04-01 of the R package R2jags (Su & Yajima 2012).

3.4 Results

The PSF responses within the introduced and native ranges varied among the *Trifolium* species (see Appendix J). As a consequence, the biogeographic differences in PSF between NZ and Europe that quantified the strength of escape from inhibitory PSF also varied considerably among the species (Figure 3.2). A number of species showed positive mean biogeographic differences, i.e. less negative PSF in NZ relative to Europe, indicating that these species escape some effects of inhibitory soil biota in NZ, and for two species (*T. campestre* and *T. micranthum)* these values were significantly more positive than zero. For most species, however, 95% confidence intervals (CI) indicated that the mean biogeographic differences were not significantly different from zero (the value that represents equal PSF responses in both ranges). Mean biogeographic differences for three out of the 11 species were slightly negative, suggesting more inhibitory PSF in NZ relative to Europe, although their 95% confidence intervals widely overlapped zero.

In contrast to expectations, the strength of escape from inhibitory PSF was not significantly correlated with the time since naturalisation of the *Trifolium* species in NZ (Figure 3.2A). While there was also no overall significant linear relationship between the strength of escape and the geographic spread of the species in NZ (Figure 3.2B), a pattern was revealed when biogeographic differences were averaged over species within two groups based on their agricultural use and geographic spread. More geographically restricted species, six out of seven of which were accidentally introduced, experienced PSF that was significantly less inhibitory in NZ relative to Europe (mean biogeographic difference = 0.73; 95% CI = 0.2, 1.3) (Figure 3.3). Conversely, widespread species, three out of four of which were intentionally introduced for agriculture, experienced the same PSF responses in both

ranges (mean = -0.01; 95% CI = -0.7, 0.7) (Figure 3.3). The strength of escape from inhibitory PSF also appeared to be similar among *Trifolium* species within the same taxonomic group (Figure 3.5), suggesting a possible phylogenetic signal.

The mean biogeographic difference in PSF averaged over all *Trifolium* species was positive, indicating a general trend in the direction of less inhibitory PSF in NZ, though this was not quite significantly different from zero (mean = 0.45; CI = -0.03, 0.97) (Figure 3.4). This trend was apparent when NZ was compared with the UK or Spain separately, though the uncertainties around the mean NZ-UK difference were much larger, reflecting wider among-species variation in the strength of escape from inhibitory PSF (Figure 3.4). Testing for correlations between the biogeographic differences in PSF and the time since naturalisation or geographic spread of the species separately for the NZ-UK and the NZ-Spain comparisons did not show any overall trends (see Appendix K).

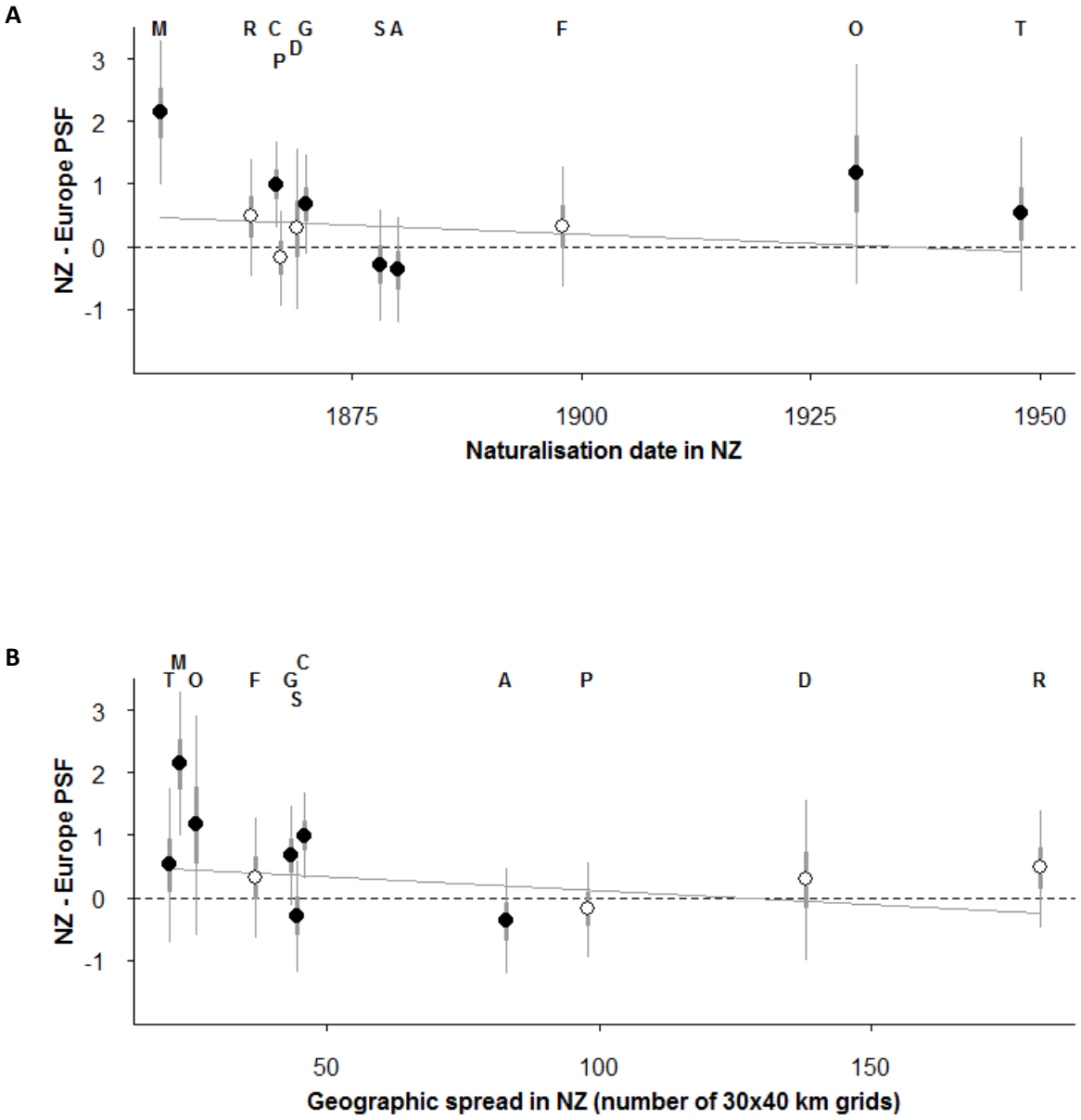

Figure 3.2. Mean biogeographic differences in plant-soil feedback (PSF) between NZ and Europe for each *Trifolium* species as a function of their: A) naturalisation date in NZ; and B) geographic spread in NZ. Bars represent 95% (thin bars) and 50% (thick bars) confidence intervals. Solid grey lines represent Bayesian linear regression model fits. The dashed line represents no difference in PSF between ranges. Positive values represent more positive PSF in NZ relative to the native range of Europe, indicating a release from inhibitory effects of soil biota, and vice-versa for negative values. Labels indicate *Trifolium* species: *T. arvense, T. campestre, T. dubium, T. fragiferum, T. glomeratum, T. micranthum, T. pratense, T. repens, T. striatum, T. ornithopodioides* and *T. tomentosum*. Symbols: ● = accidental introductions; ○ = intentional introductions. On plot A, species C and P were jittered by a value of 0.5 on the x-axis due to an identical year of naturalisation. On plot B, species G and S were jittered by a value of one grid on the x-axis due to identical spread. See Appendix K for separate comparisons between NZ and the UK or Spain.

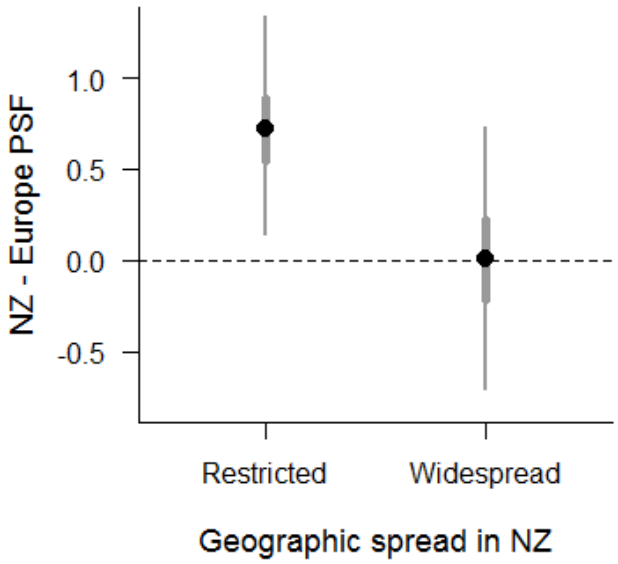

Figure 3.3. Mean biogeographic differences in plant-soil feedback between NZ and Europe for *Trifolium* species with a more restricted geographic spread in NZ (present in < 15% of 30 x 40 km grids) versus those that are widespread (present in > 15% of 30 x 40 km grids). Bars represent 95% (thin bars) and 50% (thick bars) confidence intervals. Significantly less inhibitory PSF in NZ relative to Europe is indicated by a positive biogeographic diference with 95% confidence intervals that do not overlap zero.

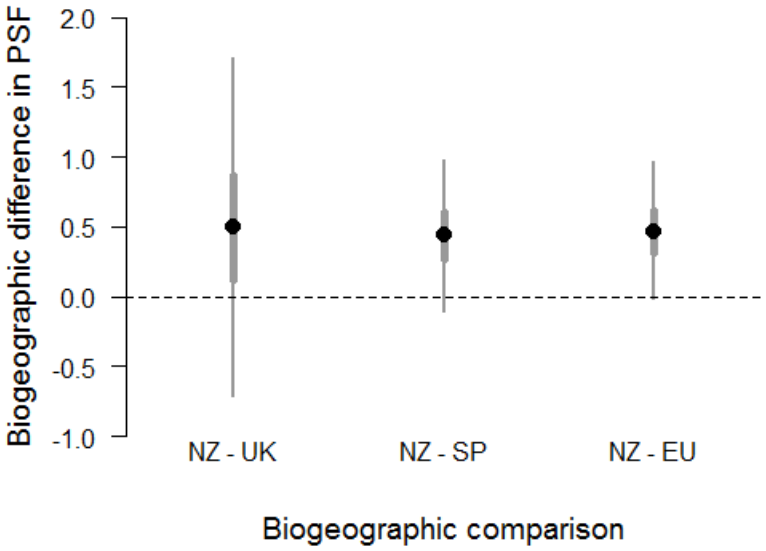

Figure 3.4. Mean biogeographic differences in plant-soil feedback averaged over all *Trifolium* species for the three biogeographical comparisons: NZ-UK (n = 9); NZ-Spain (n = 9); NZ-Europe (n = 11). Bars represent 95% (thin bars) and 50% (thick bars) confidence intervals. Significant effects sizes are indicated by 95% confidence intervals that do not overlap zero.

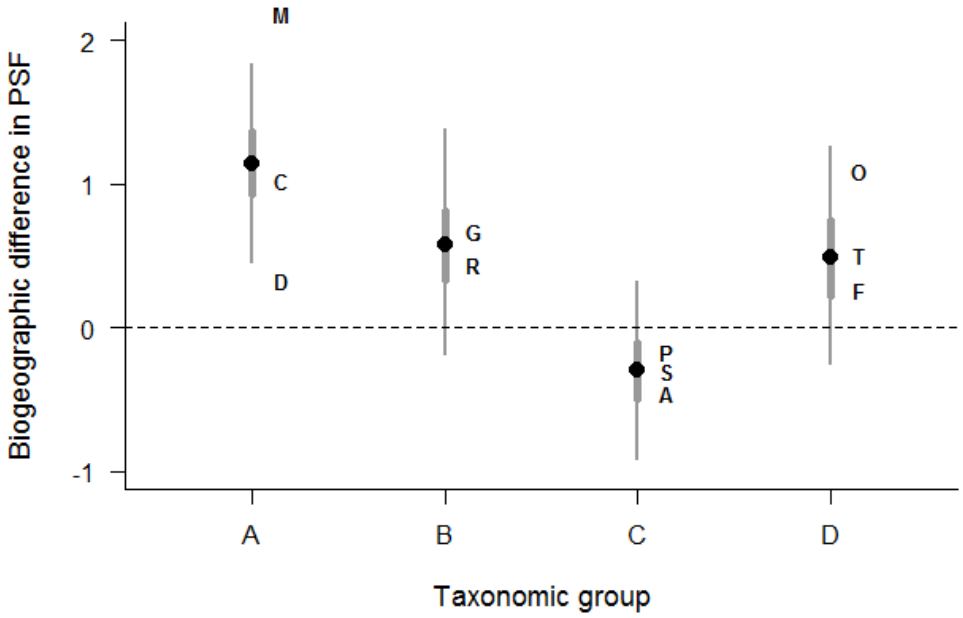

Figure 3.5. Mean biogeographic differences in plant-soil feedback between NZ and Europe within each taxonomic group of *Trifolium* (*sensu* Ellison *et al.* 2006). A = subgenus *Chronosemium*, B = section *Trifoliastrum*, C = section *Trifolium*, D = section *Vesicastrum*. Points represent mean values averaged over species within each taxonomic group, with bars showing 95% (thin bars) and 50% (thick bars) confidence intervals. Letters represent the biogeographic difference for individual *Trifolium* species before these values were averaged over species within each taxonomic group: *T. micranthum* (M), *T. campestre* (C), *T. dubium* (D), *T. glomeratum* (G), *T. repens* (R), *T. pratense* (P), *T. striatum* (S), *T. arvense* (A), *T. ornithopodioides* (O), *T. tomentosum* (T) and *T. fragiferum* (F).

3.5 Discussion

While a growing number of studies are showing that plants can escape inhibitory plant-soil feedback (PSF) in their introduced relative to their native range (e.g., Reinhart *et al.* 2003; Callaway *et al.* 2011; Maron *et al.* 2014), very few have tested whether this escape is transient, despite findings that alien plant hosts accumulate enemy species over time and as they spread (Mitchell & Power 2003; Hawkes 2007; van Kleunen & Fischer 2009; Mitchell *et al.* 2010). To date, support for the hypothesis of a transient escape from inhibitory PSF originates from studies conducted in the introduced range (Nijjer *et al.* 2007; Diez *et al.* 2010; Dostál *et al.* 2013). In contrast, we quantified the strength of biogeographic escape from inhibitory PSF (the difference in PSF between the introduced and native range) for multiple *Trifolium* species to identify whether longer-introduced and more widespread species benefit from enemy escape less.

The variation in PSF responses among the *Trifolium* species within the introduced and native range (see Appendix J) may be indicative of species-specific interactions with soil biota, possibly due to the presence of species-specific enemies or enemy densities, or alternatively, differing impacts of generalist enemies on plant performance. This suggests that PSF responses can vary among closely

related co-occurring plant species. While few have tested this, the findings of van Grunsven *et al.* (2010) showed that two species of *Tragopogon* experience different PSF responses within the same geographic region.

While our findings indicate some degree of overall biogeographic escape from inhibitory PSF in the introduced range when the responses were averaged across all *Trifolium* species (Figure 3.4), the strength of escape varied considerably among the species; some species experiencing a significant escape, while others encountering soil biota that inhibits growth as much as in the native range (Figure 3.2). Although previous biogeographic comparisons have identified that plants can escape inhibitory PSF in their introduced range (e.g., Reinhart *et al.* 2003; Callaway *et al.* 2011; Maron *et al.* 2014), others have found that PSF responses were similar in both ranges (Beckstead & Parker 2003; te Beest *et al.* 2009; Andonian *et al.* 2011; Birnbaum & Leishman 2013; Maron *et al.* 2014). It therefore appears that many alien plant species do not benefit from escaping inhibitory PSF, in line with the biotic resistance hypothesis (Maron & Vilà 2001; Levine *et al.* 2004). Our findings, however, indicate that the strength of escape from inhibitory PSF should be considered on a species-by-species basis, even among congeners that often co-occur in the same geographic region.

In contrast to Diez *et al.* (2010), our findings do not support predictions of a decline in the strength of escape from inhibitory PSF with longer residence time of the alien plant species (Figure 3.2). Although the time range of 68 years that the *Trifolium* species became naturalised was narrower than the 210 year range of species' introduction dates captured by Diez *et al*., we still observed significant differences in PSF responses among the *Trifolium* species. Given that these differences in PSF responses were not correlated with time since naturalisation, a transient escape from inhibitory PSF may not be a generalisable phenomenon.

Our results do nevertheless suggest that *Trifolium* species with a more restricted distribution, mostly introduced accidentally, are more likely to escape inhibitory PSF than more widespread, mostly agricultural species (Figure 3.3). Inhibitory PSF is driven by antagonistic organisms such as fungal and bacterial pathogens and root-feeding organisms (e.g., Mills & Bever 1998; de Deyn *et al.* 2003; van der Putten *et al.* 2005; Kardol *et al.* 2007). It is possible that the more widespread, mostly agricultural *Trifolium* species have accumulated a higher richness or density of soil-borne enemies in NZ, or have encountered more specialised enemies that do not attack the localised, accidentally introduced species. Seed of agricultural *Trifolium* species, for example, is likely to have been historically imported to NZ in far larger quantities than seed of the accidentally introduced species, providing a greater chance for native-range enemies associated with agricultural *Trifolium* to arrive in NZ.

In fact, the findings of previous studies do suggest that commercial plant species support a higher richness of pathogens than non-commercial plants (Clay 1995; Mitchell *et al.* 2010). Agricultural plant

species are also typically planted at high densities that can encourage enemy accumulation (Flory & Clay 2013 and refs. therein). Populations of these enemies present in agricultural pastures may then spill over onto conspecific naturalised populations of those agricultural plant species. However, given the links between larger geographic extent of alien plants and higher enemy richness (Hawkes 2007; van Kleunen & Fischer 2009; Mitchell *et al.* 2010), the general widespread nature of the agricultural *Trifolium* in NZ may have influenced the lack of escape from inhibitory PSF for these species. We are therefore unable to separate the effects of agricultural status and geographic spread, as was the case in previous studies (Clay 1995; Mitchell *et al.* 2010).

The lack of significant escape for many of the NZ-naturalised *Trifolium* species may have resulted from natural soil-borne enemies having caught up from Europe, or the acquisition of novel enemies in NZ. Indeed, there are many soil-borne enemies of *Trifolium* present in NZ; plant-parasitic nematodes, for example, (*Meloidogyne trifoliophila*, *M. hapla* and *Heterodera trifolii*) are prevalent (Skipp & Christensen 1983; Mercer & Miller 1997; Mercer, Bell & Yeates 2008; Zydenbos *et al.* 2011), as are a variety of soil-borne microbial pathogens (Skipp & Christensen 1983; Skipp, Christensen & Biao 1986). Clover root weevil, *Sitona lepidus* is a major agricultural pest of *Trifolium* that has been accidentally introduced to NZ from Europe (Barratt, Barker & Addison 1996; Crush & Ouyang 2007). In addition, larvae of endemic NZ beetles, *Costelytra zealandica* (grass grub) and *Pyronota* spp. (mānuka beetle) have broadened their host range to feed on clover roots and cause extensive damage to clover in NZ pastures (East & King 1977; Townsend, Nelson & Jackson 2010; Zydenbos *et al.* 2011). Despite the known presence of these enemies in NZ soils, further work is required to identify the biotic components that drive the PSF responses quantified and identify whether differences in the tolerance or susceptibility to enemies may underlie among-species variation in the PSF responses.

A number of the more restricted *Trifolium* species that experience significant release from inhibitory PSF (e.g., *T. micranthum*) have been naturalised for a similar time span as the widespread species that do not benefit from escape (Figure 3.1). The possible influence of geographic spread and agricultural status therefore appear to be independent of time since naturalisation, which does not appear to underlie variation in the strength of release among the *Trifolium* species. Indeed, the richness or impact of aboveground enemies has not always been significantly positively correlated with the residence time of alien plants species (Strong *et al.* 1977; Carpenter & Cappuccino 2005; Harvey *et al.* 2013).

The degree to which less successful alien plant species are released from enemy effects has rarely been documented (van Kleunen & Fischer 2009) because the invasion ecology literature is heavily biased towards problematic invaders (Simons 2003). If escaping inhibitory PSF is a major driver of

plant invasiveness, more invasive plant species would be expected to experience a stronger escape from inhibitory PSF. Our results indicate that more geographically restricted plant species, not considered invasive *per se*, can experience a significant escape from inhibitory PSF, questioning whether the escape from inhibitory PSF is a strong determinant of plant invasiveness. These restricted *Trifolium* species have also been present in NZ for ample time (up to 160 years) to attain wider geographic expansion. Lower propagule pressure for these species in NZ largely explains this, as a previous study has identified that the extent of intentional planting and frequency as pasture seed contaminants has strongly influenced the geographic success of NZ-naturalised *Trifolium* species (Gravuer *et al.* 2008).

Although release from inhibitory PSF is often attributed to lower impact of soil-borne enemies in the introduced range, soil-borne mutualists can also contribute to between-range differences in PSF. While we statistically removed the influence of nitrogen-fixing rhizobia from the PSF responses, arbuscular mycorrhizal fungi can also significantly enhance the growth of *Trifolium* species (Crush 1974). Generalist mutualists such as arbuscular mycorrhizae are often considered to be widely available to alien plants (Richardson *et al.* 2000a), but the assumption that NZ-naturalised *Trifolium* have access to soil-borne mutualists needs to be tested.

Lastly, the findings also indicated a possible phylogenetic signal in the degree of escape from inhibitory PSF (Figure 3.5), despite the close relatedness of the study system. This suggests that more closely related plant species may influence and respond to soil communities in comparable ways. Other studies have indicated this, for example, Brandt, Seabloom & Hosseini (2009) found that the phylogenetic distance between alien and native grasses in California was a strong determinant of PSF responses (but see Dostal & Paleckova 2011). Diez *et al.* (2010) also found that native and alien plant species within the same genus exhibited highly similar PSF responses when grown in each other's soil. The relatedness between alien and native species in recipient communities has long been predicted to influence the success of alien species (Darwin's Naturalisation Hypothesis; Darwin 1859), and a possible mechanism for this is that invaders with close native relatives are more likely to acquire native enemies that limit their success (Agrawal & Kotanen 2003; Mitchell *et al.* 2006; Strauss *et al.* 2006). Our results, however, suggest that the phylogenetic distance between alien plant species and other coexisting aliens, not just natives, may determine the strength of escape from inhibitory PSF, although direct tests of this are required.

Chapter 4: The richness and structure of arbuscular mycorrhizal fungal communities associated with *Trifolium* is similar in the naturalised and native range.

4.1 Abstract

1. Arbuscular mycorrhizal fungi (AMF) are assumed to play a minimal role in plant invasion due to the availability of widespread, generalist taxa. Recent evidence, however, suggests that many AMF taxa are not globally widespread and as a consequence, the richness and taxonomic structure of AMF communities associated with a plant species may differ between its introduced and native range, with potential plant performance implications. Despite this possibility, comparisons of AMF communities between the introduced and native range of plant species are rare.

2. We used TRFLP of the LSU-rDNA region to characterise AMF communities associated with *Trifolium arvense*, *T. repens* and *T. fragiferum* in the introduced (Banks Peninsula, New Zealand) and native (United Kingdom) range. We hypothesised that AMF alpha richness (number of taxa at the plant scale), gamma richness (number of taxa at the landscape scale) and beta diversity (differentiation in AMF community structure among plants) would be lower in NZ due to a loss of coevolved AMF. We also hypothesised that AMF community structure would differ between the ranges. A total of 63 root samples were used, originating from 10 sites in NZ and nine sites in the UK.

3. We detected 17 AMF operational taxonomic units (OTU). The findings do not support a hypothesised loss of richness in NZ, as there were no significant differences in the alpha richness, gamma richness or beta diversity of AMF taxa between NZ and the UK for each *Trifolium* species. Furthermore, geographic range (NZ/UK) and *Trifolium* host species were not significant predictors of AMF community structure, indicating a large degree of between-range and among-species overlap in AMF assemblages.

4. The availability of a variety of AMF taxa has probably contributed to the naturalisation success of these, and other, *Trifolium* species in NZ. While we cannot determine the underlying cause for the similarity in AMF community structure between the ranges, the co-introduction of European AMF or associations with cosmopolitan AMF are possible explanations.

4.2 Introduction

Interactions between plants and soil-borne organisms can enhance or constrain the performance of alien plants (Reinhart & Callaway 2006; Rout & Callaway 2012). While alien plants can benefit from escaping the inhibitory effects of belowground pathogens, parasites and herbivores (e.g., Reinhart *et al.* 2003; Callaway *et al.* 2004; Gundale *et al.* 2014; Maron *et al.* 2014), their success can be impaired by leaving behind key belowground mutualists such as rhizobia bacteria or ectomycorrhizal fungi (Richardson *et al.* 1994; Parker *et al.* 2006; Nuñez *et al.* 2009; Stanton-Geddes & Anderson 2011). The most ubiquitous soil-borne mutualists, arbuscular mycorrhizal fungi (AMF), however, are assumed to neither limit nor facilitate plant invasion (Richardson *et al.* 2000a).

Arbuscular mycorrhizal plant species commonly associate with a wide range of generalist AMF partners (Klironomos 2000) and AMF taxa have traditionally been regarded to have worldwide distributions (Morton & Bentivenga 1994). For these reasons, alien plants are assumed to acquire suitable AMF partners in new soils, and AMF availability is therefore not considered to pose a barrier to plant invasion (Richardson *et al.* 2000a; Pringle *et al.* 2009). The increasing intentional spread of AMF inocula used to enhance commercial plant productivity (Schwartz *et al.* 2006; Rosendahl, McGee & Morton 2009; Pringle *et al.* 2009), in addition to the potential inadvertent spread of AMF, suggests that plants may also be co-introduced with AMF partners from their native range. While some AMF taxa do appear to be cosmopolitan, more recent molecular evidence has suggested that a significant number of AMF taxa are more geographically restricted (Öpik *et al.* 2006, 2010; Peay *et al.* 2010). This presents the possibility that the richness and composition of AMF assemblages associated with a plant species may differ between its introduced and native range, yet there are few empirical tests of this (but see Moora *et al.* 2011).

While Moora *et al.* (2011) showed that the palm species *Trachycarpus fortuei* shared a number of cosmopolitan, generalist AMF taxa between its introduced and native range, 29% of the detected taxa were absent from the introduced range. This finding suggests that introduced plant populations may be dislocated from a proportion of their coevolved AMF partners. There is also evidence that alien plant species can evolve reduced mycorrhizal dependency (Seifert, Bever & Maron 2009), and the loss of optimal AMF partners is a potential driver for such selection. On the other hand, however, alien plants may acquire new AMF partners that are not available in native-range soils. Moora *et al.* (2011) also found potential evidence for such novel associations, as a number of AMF taxa were only detected in the introduced range.

The growth responses of plants from AMF infection vary from strongly mutualistic to strongly parasitic, depending on the taxonomic identities of both the fungal partner and the plant host (Johnson *et al.* 1997; Klironomos 2000; van der Heijden & Horton 2009). Differences in AMF

assemblages associated with plants in their introduced and native range may therefore translate to differences in plant performance, influencing the success of alien plant species (Inderjit & van der Putten 2010). Novel associations, for example, have the potential to yield highly beneficial mutualisms (Reinhart & Callaway 2006), and the dislocation from optimal mutualistic AMF taxa may result in inferior plant growth in the introduced range. Although few studies have tested for between-range differences in the plant growth effects of AMF, Callaway *et al.* (2011) found that AMF from the native range enhanced the growth of *Robinia pseudoacacia* more than AMF from the introduced range, suggesting that alien populations of this species may have been dislocated from optimal AMF mutualists. A number of studies within invaded plant communities have additionally shown that the competitive ability of invasive relative to co-occurring plant species is highly dependent on the taxonomic identities of the AMF available in the soil (Bray *et al.* 2003; Stampe & Daehler 2003; Carey *et al.* 2004; Shah *et al.* 2008).

Increasing the richness of mycorrhizal assemblages has also been shown to enhance the productivity of plant communities by capturing nutrients more effectively, but with relationships that vary depending on the plant and mycorrhizal species present, in addition to the nutrient content of the soil (van der Heijden *et al.* 1998b; Jonsson *et al.* 2001). The success of plant invaders may therefore be influenced by both the identities and richness of AMF taxa available in new soils. Despite this, however, further studies using a biogeographical approach (Hierro *et al.* 2005) to compare AMF communities between the introduced and native ranges of plant species are lacking.

In this study, we compare the richness and community structure of AMF associated with three *Trifolium* (clover) species in their introduced (New Zealand) and native (United Kingdom) range. *Trifolium* species form a particularly important component of agricultural systems in New Zealand (NZ) (Caradus *et al.* 1996), all of which are introduced, because NZ lacks native *Trifolium*. While experiments in the 1970s showed that AMF resident in NZ soils were compatible and improved the growth of the agricultural species *Trifolium repens*, introduced AMF strains were shown to provide larger growth benefits (Powell 1976, 1977a; b, 1979). These experiments also represent the intentional introduction of the AMF species *Glomus tenuis* and *Gigaspora margarita* to NZ (Powell 1979). The intentional inoculation of agricultural *Trifolium* with AMF in NZ has seemingly been very limited, despite the availability of commercial AMF preparations suitable for a range of plant species. Strict biosecurity regulations limit the additional introduction of inoculant containing AMF species apparently not yet present in NZ. Nevertheless, *Trifolium* may have been inadvertently co-introduced with AMF taxa from their native range, given that biological materials, potentially harbouring hyphae or spores, were extensively imported to NZ by European settlers from the mid-19th century onwards (Allen & Lee 2006).

Although *Trifolium* associate with AMF facultatively (Kendall & Stringer 1985), AMF can be particularly beneficial to the performance of legume species. AMF associations not only benefit legume plants directly by increasing water and phosphorus availability, but also indirectly by enhancing the level of root nodulation by nitrogen-fixing rhizobia (Crush 1974; Chalk *et al.* 2006; Abd-Alla *et al.* 2014). The development of root nodules can even fail in the absence of AMF infection due to a lack of plant-available phosphorus (Mosse, Powell & Hayman 1976). As a consequence, the competitive ability of *Trifolium* species relative to plant competitors, such as grass species, can be greatly enhanced by AMF (Crush 1974; Buwalda 1980) and the availability of AMF is also therefore likely to facilitate the naturalisation of legume species outside of cultivated areas.

We used three NZ-naturalised *Trifolium* species to test the following hypotheses:

1. The alpha and gamma richness and beta diversity of AMF taxa available to *Trifolium* is lower in the introduced than in the native range.

2. The community structure of AMF associated with *Trifolium* differs between the introduced and native range.

4.3 Methods

4.3.1 Study species and collection of root material

Three European *Trifolium* species that became naturalised in New Zealand (NZ) between 150-116 years ago (Gravuer *et al.* 2008) were used for the study: *T. repens* (a perennial), *T. fragiferum* (a perennial) and *T. arvense* (an annual). *T. repens* has been extensively planted in NZ as an integral component of agricultural pastures (Caradus *et al.* 1996); *T. fragiferum* was also intentionally introduced but agricultural planting has been more limited (Gravuer 2004); and although *T. arvense* arrived accidentally, it is now one of the most widespread *Trifolium* species in NZ (Gravuer 2004). These species were selected based on differences in their habitat preferences that may be associated with differences in AMF associations, with the aim of detecting a variety of AMF taxa in both ranges. *T. fragiferum* is adapted to waterlogged and saline soils (Townsend 1985), *T. arvense* is adapted to dry, infertile and sandy soils (Knight 1985), and *T. repens* is less habitat-restricted, but does not perform well in dry soils (Burdon 1983).

Given the extensive introduction of plant species to NZ from the United Kingdom (UK) (Allen & Lee 2006) that may have harboured European AMF, the UK was chosen as a representative native region of the *Trifolium* study species to sample from. Three to five naturalised populations of each *Trifolium* species were located excluding agricultural pastures in NZ (Banks Peninsula) and in the UK, spanning sites with a range of habitat and soil types (Table L.1, Appendix L). Three to four plants at a distance

of at least 8 m apart were randomly chosen at each site and dug up with intact root systems. Roots were thoroughly washed with tap water, sections of root with root tips removed and freeze- (NZ) or air-dried (UK) for 24 h before being stored in vials with silica gel desiccant.

4.3.2 Molecular methods

We used Terminal Restriction Fragment Length Polymorphism (TRFLP) (Liu & Marsh 1997) to characterise AMF communities in *Trifolium* root samples. In contrast to the polyphyletic ectomycorrhizae, AMF consist of a monophyletic clade (Glomeromycota) (Schüβler, Schwarzott & Walker 2001), which has allowed development of putative AMF-specific primers that avoid the amplification of other soil fungal taxa. Total DNA was extracted from root samples using a DNeasy Plant Mini Kit (Qiagen) with a bead-beating system, following the manufacturer's protocol. DNA extractions were quantified using a NanoDrop 2000 and were repeated if samples had a concentration below 10ng/µl. There were 63 successful DNA extractions, which included 8-15 samples originating from 3-5 field sites for each species in each range (Table L.2, Appendix L). The samples originated from a total of 19 different field sites (9 in the UK and 10 in NZ).

We used a nested-PCR and enzymatic restriction protocol adapted from Van de Voorde *et al.* (2010) that amplified the 5'-end of the large subunit (LSU) rRNA gene. PCR was first performed with the universal fungal primers LR1 (5'-GCATATCAATAAGCGGAGGA-3') and FLR2 (5'-GTCGTTTAAAGCCATTACGT-3') (van Tuinen *et al.* 1998; Trouvelot *et al.* 1999). Product from this first reaction was then amplified with the fluorescently-labelled primers FAM-FLR3 (5'-GTTGAAAGGGAAACGRTTRAAG-3') and NED-FLR4 (5'-ATTACGTCAACATCCTTA-3') (Gollotte, van Tuinen & Atkinson 2004). This primer system is specific to Class Glomeromycota, allowing the discrimination of AMF taxa, although the approach has been shown to miss certain AMF lineages and result in a bias towards the Glomeraceae family (Krüger *et al.* 2009; Kohout *et al.* 2014). The first PCR reaction contained: 12.75 µl milli-Q water, 2.5 µl 10x Fast Start High Fidelity Reaction Buffer (Roche Diagnostics), 1 µl $MgCl_2$ (25 mM), 2.5 µl of each primer (concentration of 10 µM), 2.5 µl dNTP mix (concentration of 2 mM each), 0.125 µl BLOTTO (10% w/v fat-free milk powder), 0.125 µl Taq polymerase (5 U/µl, HotStarTaq Plus, Qiagen) and 1 µl template DNA. Thermal cycling conditions were: 5 min at 95° C, then 35 cycles of 30 sec at 94° C, 40 sec at 58° C and 70 sec at 72° C, followed by 7 min at 72° C before cooling. The second PCR reaction contained: 11.88 µl milli-Q water, 2.5 µl Fast Start High Fidelity Reaction Buffer with $MgCl_2$ (Roche Diagnostics) (10 µM), 1µl $MgCl_2$ (25 mM), 1.8 µl of primer FAM-FLR3 (10 µM), 2.5 µl of primer NED-FLR4 (10 µM), 0.7 µl of primer FLR3 (10 µM), 2.5 µl dNTP mix (10 µM), 0.125 µL Taq polymerase (5 U/µl, HotStarTaq Plus, Qiagen) and 2 µl 1:100 parts diluted PCR product from the first reaction. Thermal cycling conditions were: 5 min at 95° C, then 27 cycles of 30 sec at 94° C, 40 sec at 54° C and 60 sec at 72° C, followed by 15 min at 72° C before

cooling. PCR product concentration was verified using 1.5% agarose gel electrophoresis. The two restriction enzymes AluI and MboI (New England Biolabs, Ipswich, MA, USA) were used to digest the second-round PCR product. A mixture containing 3.5 µl ddH_2O, 1 µl buffer, 0.1 µl bovine serum albumin, 5 µl PCR product and 0.4 µl of the restriction enzymes AluI and MboI was incubated at 37° C for 3 h, after which enzymes were inactivated by heating to 94° C for 3 min. Restriction products were purified using ethanol precipitation.

Fragments were size-fractionated on an automated 3130 Genetic Analyser sequencer using the GeneScan-500 LIZ system as a size standard (Applied Biosystems, Carlsbad, CA, USA). Samples that were over- or under-loaded (highest peak > 80 000 or < 1000 relative fluorescence units respectively) were re-run at an adjusted concentration. This generated four electrophoreograms per root sample, representing separate peak-profiles for the forward- and reverse-end fragments from the two restriction enzyme digests.

4.3.3 Statistical analysis

Data processing and statistical analyses were performed in R version 3.0.1 (R Development Core Team 2013). Terminal restriction fragments (TRF) < 50 base pairs in length were removed to eliminate primer-dimer artifacts. TRF with an electrophoreogram peak height < 30% of the highest peak within each sample were also removed to eliminate signals with low florescence. The R package TRAMPR (Fitzjohn & Dickie 2007) was used assign true TRF peaks into operational taxonomic units (OTU) and search for OTU matches across the root samples. OTU assignments required peaks to be present in all four enzyme-primer combinations per root sample with a minimum ratio of 1.2 from the maximum to second highest peak within root samples. Matches of OTU across root samples required peaks to be present within a 3 base pair margin. OTU assignments in each sample were visually inspected on electrophoreograms and clearly-missed OTU were added before an OTU by sample presence-absence matrix was generated.

Alpha richness of AMF taxa was quantified by calculating the mean number of OTU observed per plant (root sample) for each *Trifolium* species in each range, with 95% confidence intervals calculated by: mean ± 1.96 x standard error. The number of plants and field sites sampled per species in each range varied (8-15 plants from 3-5 field sites; Table L.2, Appendix L) and this confounded the total number of OTU observed per species in each range (Figure L.1, Appendix L). Sampling effort therefore needed to be accounted for when OTU gamma richness (number of OTU per species in each range) was quantified. We achieved this by randomly assessing the number of OTU occurring in an equal number of plants (eight) originating from an equal number of field sites (three) 10,000 times for each species in each range. Mean gamma richness for each species in each range was then calculated from the 10,000 estimates, with 95% confidence intervals determined from the 2.5 and

97.5% quantiles of the 10,000 estimates. Beta diversity was quantified following Anderson, Ellingsen & McArdle (2006) by assessing the multivariate homogeneity of dispersion among samples, which measured the differentiation in AMF community structure among plants of each *Trifolium* species within each range. This was performed by calculating the mean distance to the centroid of samples, based on Raup-Crick distances for 10,000 simulations (the functions raup-crick and betadisper in version 2.0-10 of R package vegan (Chase *et al.* 2011; Oksanen *et al.* 2013)). We expected AMF communities associated with each species to be more homogeneous in NZ than in the UK due to the presence of fewer suitable AMF taxa in NZ, and we tested for significant between-range differences in beta diversity for each species using Tukey's Honest Significant Difference (HSD) (TukeyHSD function of R package vegan (Oksanen *et al.* 2013)). Given that TRFLP may not be able to separate every AMF species (Dickie & FitzJohn 2007; Kohout *et al.* 2014) and the number of AMF taxa detected is contingent upon sampling effort, our quantification of AMF richness and beta diversity provides a relative metric within the study, rather than absolute values.

Non-metric multidimensional scaling (NMDS) ordination was performed to visualise AMF community structure as a function of range (NZ/UK) and *Trifolium* species (metaMDS function with two dimensions, Jaccard distance measure and a maximum of 10,000 iterations in R package vegan). This ordination produced a stress value (0.052) and a nonmetric R^2 value between ordination distance and observed dissimilarity (0.997) that indicated a very good ordination representation in two dimensions. Permutational multivariate analysis of variance (PER-MANOVA) (Anderson 2001) was performed to test whether range, *Trifolium* species or field site significantly influenced AMF community structure (adonis function with Jaccard distance and 1000 permutations in R package vegan). Separate PER-MANOVA tests were performed for each variable (range, *Trifolium* and field site) and permutations were constrained within field sites when the effects of range and *Trifolium* species were tested, so that similarity among root samples from each site did not influence tests for between-range and among-species differences. Two 'singleton' OTU that were present in only one root sample and thus mathematically uninformative were excluded from multivariate analyses, resulting in the loss of two root samples.

We additionally used the peak-profile method (Dickie & FitzJohn 2007) to analyse the TRFLP data. To do so, complete linkage hierarchical clustering was performed to remove noise in the data by clustering TRFLP peaks within four base pair margins per primer-enzyme combination and root sample (see Peay *et al.* 2013). Richness and diversity calculations were then repeated with this clustered data. This showed that the peak-profile approach produced very similar results as assigning peaks as OTU using the R package TRAMPR; we therefore only present results from the TRAMPR method.

4.4 Results

A total of 17 arbuscular mycorrhizal OTU were distinguished, each present in one to 34 root samples. For each *Trifolium* species per range, AMF alpha richness (the number of OTU per plant) varied between 1.5 - 2.5 and gamma richness (the number of OTU in each range) varied between 6 - 9.8, but there were no significant differences in richness between NZ and the UK for each *Trifolium* species (Figure 4.1, Figure 4.2). There was also no significant difference in AMF beta diversity between the ranges for each *Trifolium* species (Table 4.1), indicating a similar degree of differentiation in AMF community structure among plants in both ranges.

The structure of AMF communities was similar between the ranges, and also among the three *Trifolium* species (Figure 4.3). PER-MANOVA tests indicated that the field site origin of root samples significantly influenced community structure ($r^2 = 0.46$, $p = 0.001$, Table 4.2), as expected, given that root samples from the same site are likely to be more similar. When this field site similarity was accounted for by constraining permutations within sites, PER-MANOVA showed that AMF community structure was not significantly different among *Trifolium* species or between the ranges ($r^2 = 0.07$, 0.03; $p = 0.2$, 0.9; Table 4.2).

Examining the raw OTU data showed that seven of the OTU were detected in roots of all three *Trifolium* species (Figure L.2, Appendix L). A total of 10 OTU were detected in the UK, all of which also occurred in NZ (Figure L.2, Appendix L). The remaining seven OTU were only found in NZ; however, this could be a sampling artifact, given that more plants were sampled in NZ. Plotting the number of OTU against the number of root samples per *Trifolium* species in each range showed that the number of OTU detected did not level-out at the highest sampling effort, and thus, our sampling effort did not capture all OTU in each range (Figure L.1, Appendix L).

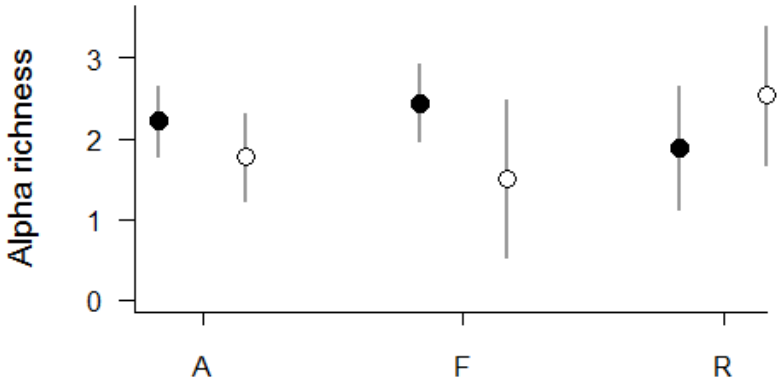

Figure 4.1. Alpha richness of arbuscular mycorrhizal OTU (mean number of OTU per plant) for each *Trifolium* species (A = *T. arvense*, F = *T. fragiferum*, R = *T. repens*) in the native range of UK (black points) versus the introduced range of NZ (white points) according to TRFLP data. Bars represent 95% confidence intervals.

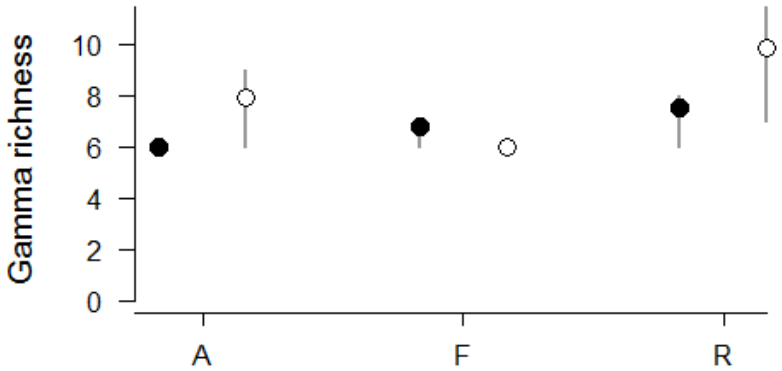

Figure 4.2. Gamma richness of arbuscular mycorrhizal OTU (mean maximum number of OTU, statistically corrected for sampling effort) for each *Trifolium* species (A = *T. arvense*, F = *T. fragiferum*, R = *T. repens*) in the UK (black points) versus NZ (white points) according to TRFLP data. Bars represent 95% confidence intervals.

Table 4.1. AMF beta diversity for each *Trifolium* species in each range (measured as mean distance to group centroid using Raup-Crick distances with functions raup-crick and betadisper of R package vegan (Oksanen *et al.* 2013)). Tests for significant differences between NZ and the UK for each species were performed using Tukey's HSD test (TukeyHSD function in R package vegan).

Species	Mean distance to group centroid (UK)	Mean distance to group centroid (NZ)	Difference between UK and NZ	Lower CI	Upper CI	Adjusted P value
T. arvense	0.24	0.17	0.07	-0.06	0.20	0.59
T. fragiferum	0.15	0.03	0.13	-0.02	0.28	0.11
T. repens	0.17	0.23	-0.05	-0.18	0.07	0.79

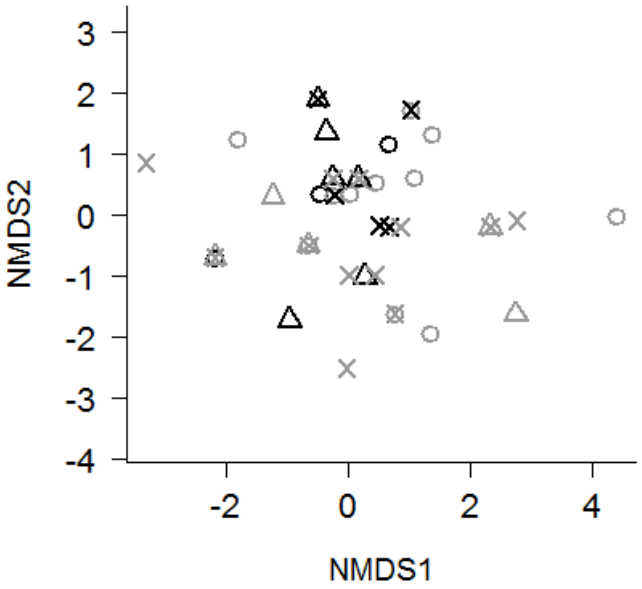

Figure 4.3. Non-metric multidimensional scaling (NMDS) ordination of AMF community structure according to TRFLP data, with colours representing geographic range (UK = black, NZ = grey) and symbols representing *Trifolium* host species (*T. arvense* = circle, *T. fragiferum* = triangle, *T. repens* = cross). Community structure was not significantly different between the ranges or among the *Trifolium* species according to PER-MANOVA tests (Table 4.2).

Table 4.2. Output statistics from PER-MANOVA (adonis function in R package vegan; Oksanen *et al.* 2013) used to test whether the range (NZ/UK), *Trifolium* host species, or field site origin of root samples significantly influenced AMF community structure.

	Degrees of freedom	Sequential sum of squares	Mean squares	F statistic	Partial R^2	p value
Range (NZ/UK)	1	0.78	0.78	2.12	0.03	0.9
Residuals	59	21.63	0.37	0.97		
***Trifolium* spp.**	2	1.60	0.80	2.22	0.07	0.239
Residuals	58	20.81	0.36	0.93		
Field site	18	10.34	0.57	2.00	0.46	0.001
Residuals	42	12.10	0.29	0.54		

4.5 Discussion

While alien plants are generally assumed to acquire compatible AMF partners in recipient soils (Pringle *et al.* 2009, and refs. therein), the richness and taxonomic structure of AMF communities available in the introduced range may differ from the native range. Such biogeographic differences in AMF associations have the potential to affect alien plant performance and influence plant invader success, but few studies have compared AMF richness and community structure between the introduced and native range of a plant species.

Our results indicate that all three *Trifolium* species studied have access to a similar number of AMF taxa in their introduced (NZ) as in their native (UK) range, both at the plant (alpha richness) and landscape (gamma richness) scale. The differentiation in AMF community structure among plants of each species in each range (beta diversity) was also similar. These findings therefore do not support the hypothesis that *Trifolium* have been dislocated from compatible AMF mutualists in NZ. Given that AMF can greatly enhance the growth and competitive ability of legumes (Crush 1974; Chalk *et al.* 2006; Abd-Alla *et al.* 2014), the availability of a variety of compatible AMF taxa in NZ soils has probably facilitated the successful naturalisation of these *Trifolium* species outside of cultivated areas, and has probably also contributed to the agricultural productivity of *Trifolium* in NZ. Direct quantification of the growth benefits of AMF in both ranges would, however, be necessary to elucidate whether AMF have an equal effect on *Trifolium* growth in Europe and NZ.

Furthermore, the similarity of AMF community structure between the introduced and native range suggests that NZ-naturalised *Trifolium* may have access to coevolved AMF partners. The presence of cosmopolitan AMF taxa that are native to both the UK and NZ may explain this pattern, as a number of AMF taxa are indeed known to be globally widespread (Öpik *et al.* 2006). However, there is also evidence that major geographical barriers can restrict AMF dispersal (Kivlin, Hawkes & Treseder 2011) and thus, the cosmopolitan distributions of some AMF taxa may have resulted from human-mediated transport. The distribution of the common AMF taxa, *Glomus mosseae*, for example, appears to be consistent with human-mediated dispersal coinciding with the spread of intensive agriculture (Rosendahl *et al.* 2009). It is therefore possible that the similarity of AMF communities between NZ and the UK is the result of co-introduction of *Trifolium* with European AMF. Indeed, potted plants with intact root systems were even introduced to NZ from the UK (e.g., Herriott 1919), and it is therefore probable that propagules of European AMF have been transported to NZ. The *Trifolium* species studied have also been naturalised in NZ for between 116-150 years, providing ample time for European AMF partners to be subsequently introduced and accumulate.

The origins of mycorrhizal taxa are, however, difficult to substantiate (Nuñez & Dickie 2013) and other possible explanations for the between-range similarity in AMF community structure are that AMF have been introduced to the UK from NZ, or introduced to both the UK and NZ from another provenance. Furthermore, while a considerable number of other studies have used the same primers as our analysis (e.g., Van de Voorde *et al.* 2010; Krishnamoorthy *et al.* 2014; Wetzel *et al.* 2014), these primers may not be able to distinguish all AMF species, and may also miss rare AMF taxa (Kohout *et al.* 2014). DNA sequence data is thus required to elucidate whether the OTU occurring in both NZ and the UK actually represent the same AMF species, or a higher taxonomic level such as the same AMF genus.

We cannot rule out the possibility that the AMF taxa detected in NZ but not in the UK (Figure L.2A, Appendix L) represent novel associations that do not occur in the native range (Reinhart & Callaway 2006); however, they may have been detected if sampling was increased in the UK. The lack of significant between-range difference in AMF richness after sampling effort had been accounted for argues against the occurrence of novel associations in NZ. Once more, DNA sequence data would help to elucidate whether the AMF taxa unique to NZ samples have been reported as present in Europe and establish the likelihood of them representing novel associations.

The similarity in AMF community structure among the three *Trifolium* species (Figure 4.3; Figure L.2, Appendix L) is perhaps not surprising, given their close phylogenetic relatedness and the lack of specificity in plant-AMF associations in general (e.g., Klironomos 2000). Pivato *et al.* (2007), for example, also showed that four species in the closely related legume genus *Medicago* had highly similar AMF assemblages within their native range. The AMF taxa detected in roots of only one *Trifolium* species in this study likely represent site-specific or less common taxa, as opposed to AMF taxa that are plant species-specific. This lack of host-specificity suggests that the AMF communities associated with widespread agricultural *Trifolium* may have facilitated the naturalisation of accidentally introduced *Trifolium* species in NZ. Nevertheless, plant species have been shown to preferentially associate with certain AMF taxa (Zhang *et al.* 2010; Helgason *et al.* 2014), and it is possible that non-European *Trifolium* species of American or African provenance preferentially associate with different AMF taxa that are not available in NZ.

Despite the general assumption that alien plants can acquire suitable AMF partners, the establishment of arbuscular mycorrhizal plant species may be limited by low AMF inoculum in certain environments in NZ. The invasion of *Hieracium* into forests dominated by the native ectomycorrhizal tree species *Nothofagus* in NZ, for example, has been shown to be limited by low AMF abundance in the soil (Spence *et al.* 2011). This suggests that low AMF inoculum may also limit the performance of arbuscular mycorrhizal plant species in soils that have been recently cleared from native NZ scrub dominated by ectomycorrhizal mānuka and kānuka (*Leptospermum* and *Kunzea* spp.). The conversion of such common native ectomycorrhizal vegetation in NZ into agricultural pastures dominated by arbuscular mycorrhizal European grass and *Trifolium* species may therefore have facilitated the development AMF-rich soil communities that are amenable to the naturalisation or invasion of many other alien arbuscular mycorrhizal plant species.

Chapter 5: Rhizobia associated with *Trifolium* in the naturalised and native range exhibit a similar strain richness and nodulation ability.

5.1 Abstract

1. Although introduced plants can benefit from escaping natural enemies, their success can be impaired by losing key mutualists such as nitrogen-fixing bacteria (rhizobia). The compatibility and symbiotic effectiveness of rhizobia strains varies among *Trifolium* (clover) species, so populations of introduced clover rhizobia may facilitate the growth of some *Trifolium* species, but be less effective or even parasitic to others.

2. We compared the nodulation ability and strain richness of rhizobia associated with seven European *Trifolium* (clover) species in their introduced (New Zealand) and native (United Kingdom) ranges by conducting a glasshouse experiment, genetic fingerprinting (rep-PCR with ERIC primers) and phylogenetic analysis of the symbiotic nodD gene. We hypothesised that clover rhizobia in NZ soil would be optimal for agricultural perennial *Trifolium* species, but be less effective for annual *Trifolium* relative to rhizobia strains available in UK soils. We also expected clover rhizobia to exhibit lower strain richness in NZ relative to the UK, due to the introduction of a limited number of strains.

3. The nodulation ability of rhizobia from NZ was similar to strains from the UK for all seven *Trifolium* species tested, indicating that access to effective nitrogen-fixing rhizobia has not constrained the success of these species in NZ. There was also no evidence for regional scale adaptation between *Trifolium* and rhizobia populations from the same provenance (NZ/UK). Genetic fingerprinting indicated that the strain richness of clover rhizobia is high in NZ soils and comparable to the richness of clover rhizobia populations in the UK. Furthermore, the nodD phylogenetic analysis showed that NZ rhizobia genotypes were not distinct from UK genotypes, indicating that they probably originated from Europe.

4. Our results indicate that the co-introduction of rhizobia with alien legume hosts, probably both inadvertently and intentionally as agricultural inoculants, can result in diverse populations of introduced rhizobia that facilitate legume naturalisation.

5.2 Introduction

Alien plant species can benefit from escaping the negative effects of natural soil-borne enemies (e.g., Reinhart *et al.* 2003; Callaway *et al.* 2004; Gundale *et al.* 2014; Maron *et al.* 2014), but an absence of suitable soil-borne mutualists in new soil environments can constrain their success (Mitchell *et al.* 2006). Legumes, for example, perform very poorly in soils that lack suitable nitrogen-fixing bacteria (rhizobia) (Parker 2001). While some alien legume species can acquire novel rhizobia associations by utilising local strains (Pérez-Fernández & Lamont 2003; Ndlovu *et al.* 2013), many plant-rhizobia symbioses are highly specific (Wang *et al.* 2012), and thus, many alien legumes rely on the availability of coevolved rhizobia.

Commercial legume species are often intentionally co-introduced with suitable rhizobia to facilitate their establishment in new environments (Richardson *et al.* 2000a). Further introductions of non-native rhizobia also appear to have inadvertently resulted from human-mediated transport, such as on the surface of plant seeds (Pérez-Ramírez *et al.* 1998). As a consequence, many alien legumes do have access to coevolved rhizobia partners that facilitate their invasion (Stepkowski *et al.* 2005; Wei *et al.* 2009; Rodríguez-Echeverría 2010; Andrus *et al.* 2012; Ndlovu *et al.* 2013; Crisóstomo *et al.* 2013). In many other cases, though, a lack of suitable rhizobia in new soils has been shown to limit the establishment and spread of alien legumes (Parker 2001; Parker *et al.* 2006; Stanton-Geddes & Anderson 2011; Wandrag *et al.* 2013). Some introduced rhizobia populations also exhibit low genetic diversity (Seguin *et al.* 2001; Riah *et al.* 2014; Junier *et al.* 2014), consistent with the occurrence of a genetic bottleneck resulting from the introduction of a limited number of founder bacterial strains.

The degree to which rhizobia promotes plant growth varies greatly among bacterial strains (Bever *et al.* 2013), and rhizobia can even parasitise the host plant (Denison & Kiers 2004; Pryor *et al.* 2004). The availability of compatible rhizobia therefore does not necessarily represent a highly beneficial symbiosis for the plant. Moreover, between-range differences in the taxonomic identity of rhizobia strains, such as novel associations in the introduced range, may not actually be associated with functional differences in symbiotic effectiveness (Parker, Wurtz & Paynter 2007; Birnbaum *et al.* 2012). In order to fully understand the role of rhizobia in plant invasion, it is therefore important to test for differences in the symbiotic effectiveness and taxonomic identities of rhizobia between the introduced and native range.

Comparisons of rhizobia associated with alien legumes in their introduced and native range have largely been focussed on woody species such as *Acacia, Cytisus*, *Mimosa* and *Robinia* (Lafay & Burdon 2006; Parker *et al.* 2007; Callaway *et al.* 2011; Rodríguez-Echeverría *et al.* 2011; Birnbaum *et al.* 2012; Ndlovu *et al.* 2013; Crisóstomo *et al.* 2013; Wandrag *et al.* 2013). These species are capable of forming effective nitrogen-fixing symbioses with a variety of rhizobia taxa and are thus more likely to

be able to utilise native rhizobia in their introduced range. In contrast, the rhizobia associations of *Trifolium* (clover) are confined to *Rhizobium leguminosarum* biovar *trifolii* (Burton 1985), with further strain specificity among species of *Trifolium* (e.g., Howieson *et al.* 2005). Clover species have been widely introduced around the world for agricultural use, including to regions that lack compatible native rhizobia, such as New Zealand (NZ) (Greenwood 1976; Greenwood & Pankhurst 1977; Jarvis *et al.* 1977). Clover rhizobia are now nevertheless widespread in NZ soils (Lowther & Kerr 2011) following their likely inadvertent introduction by European settlers in the mid-19th century (Greenwood 1976) and the use of commercially-developed inoculant for over 60 years (Lowther & Kerr 2011). Given that the compatibility and symbiotic effectiveness of specific rhizobia strains varies greatly among species of *Trifolium* (Howieson *et al.* 2005; Melino *et al.* 2012), naturalised populations of clover rhizobia may be conducive to the growth of some *Trifolium* species in NZ, but remain limiting to congeners.

Not all *Trifolium* species present in New Zealand were introduced for agricultural purposes; 16 of the 25 *Trifolium* species currently naturalised in NZ were introduced accidentally, most of which are annuals (Gravuer *et al.* 2008). Naturalised clover rhizobia in NZ may largely originate from commercial strains selected for high nitrogen fixation with agricultural *Trifolium* species, such as the extensively-planted perennials *T. repens* and *T. pratense*. Such agricultural clover rhizobia strains may not be highly symbiotic or even compatible with other *Trifolium* species, particularly annuals (Beauregard *et al.* 2003; Yates *et al.* 2005; Nangul *et al.* 2013). Consequently, it is possible that the performance of accidentally introduced annual *Trifolium* species is limited by an absence of optimal coevolved rhizobia in NZ. Indeed, naturalised rhizobia populations have been shown to be sub-optimal for the growth of a number of European annual *Trifolium* species in regions of Australia (Denton *et al.* 2000; Drew *et al.* 2011).

The plant growth benefits provided by specific rhizobia strains can additionally depend on the genotype of the legume species (Jones & Hardarson 1979; Burton 1985; Smith & Goodman 1999; Heath & Tiffin 2007). There is also evidence that legume populations can perform best with locally coexisting rhizobia relative to strains isolated from the same legume species elsewhere (Sherwood & Masterson 1974; Mytton 1975; Lie *et al.* 1987; Chanway, Holl & Turkington 1989; but see Barrett *et al.* 2012). This suggests that alien legume populations may, over time, adapt to the rhizobia available, whereby legume genotypes benefiting most from the rhizobia strains available are more likely to be selected. Such specificity in legume growth responses depending on the provenance of the plant and rhizobia population has rarely been considered in context of plant invasion. Few studies examining the symbiotic effectiveness of rhizobia between the introduced and native range, for example, have used seed from plant populations in both ranges (but see Birnbaum *et al.* 2012; Birnbaum &

Leishman 2013), and fewer still have combined this with an examination of the genetic relatedness or strain richness of rhizobia in both ranges.

We tested the following hypotheses:

1. The nodulation ability and plant growth effects of root nodule bacteria do not differ between NZ and the UK for perennial (agricultural) *Trifolium* species, but are lower in NZ for annual *Trifolium* species.

2. *Trifolium* seed of NZ or UK provenance grows best with rhizobia from the same provenance.

3. The strain richness of *Trifolium*-associated rhizobia is lower in NZ than in the UK.

5.3 Methods

5.3.1 Study species and root nodule collection

We studied seven European *Trifolium* species that have been naturalised in New Zealand for at least 84 years, comprising two common agricultural perennial species (*T. pratense, T. repens*) and five naturalised annual species (*T. arvense, T. campestre, T. dubium, T. ornithopodioides, T. striatum*). All annuals were accidentally introduced, apart from *T. dubium*, which has not been extensively used commercially (Boswell *et al.* 2003). *Trifolium* species can be categorised into rhizobia 'effectiveness groups' based on the compatibility and growth benefits of various strains of clover rhizobia (Burton 1985 and refs. therein). The annuals *T. arvense* and *T. glomeratum* are in a different effectiveness group than the agricultural perennial species *T. repens* and *T. pratense*, suggesting that the clover rhizobia widespread in NZ soil may not be optimal for these species. The effectiveness groups of the other *Trifolium* study species are unknown.

Plants were collected at five sites per species in each range: from South Wales and the southern England in the United Kingdom (UK) in June 2013; and from Banks Peninsula to Timaru in the South Island of New Zealand in December 2013. The sampling sites consisted of naturalised clover populations excluding managed pastures, and these sites covered a similar geographic extent in the two ranges: from 150-230 km depending on species (see Appendix M). Three plants at least 4 m apart were randomly selected for sampling at each field site for a total of 15 plants per species per range. Roots of each plant were thoroughly washed and nodule samples were removed and preserved for up to seven months in vials with silica gel desiccant.

5.3.2 Bacteria isolation

Desiccated nodules were rehydrated at 4° C in de-ionised (DI) water overnight. Rehydrated nodules were surface-sterilised with a 30 sec immersion in 95% ethanol, a 2 min immersion in 20% household

sodium hypochlorite with a few drops of Tween 20 surfactant and then rinsed thoroughly in three changes of sterile DI water. A single nodule from each plant was crushed with a sterile glass rod and the exudate streaked onto plates of yeast mannitol agar (YMA; Table N.1, Appendix N). Plates were incubated at 20° C for 2-5 days to attain sufficient growth of well-formed individual bacterial colonies. From each plate, single individual colonies resembling the morphology of rhizobia (opaque, glistening, milk-white colour with entire margin (Somasegaran & Hoben 1994)) were re-streaked onto YMA plates, cultured for 2 days at 20° C and then stored short term for up to two weeks at 4° C . When morphological differences indicated multiple colony types were still present on a plate, further rounds of streak plates were performed to obtain a single colony isolate per nodule. Glycerol stocks were prepared for longer-term storage by culturing isolates in 1 ml of yeast mannitol broth (YMB; Table N.2, Appendix N) for 24 h on a rotary shaker at 28° C and 120 revolutions per min (rpm), to which an equal volume of sterile 60% glycerol was added before storing at -80° C .

5.3.3 DNA extraction

Each isolate was cultured in 1 ml of YMB on a rotary shaker at 28° C and 120 rpm for 24 h. Broth cultures were centrifuged at 16000 x g and the supernatant discarded to leave bacterial cell pellets, from which DNA was extracted according to the manufacturer's instructions using the GenElute™ Bacterial Genomic DNA extraction kit (Sigma Aldrich). The DNA quantity in each extraction was verified using 1% agarose gel electrophoresis.

5.3.4 DNA fingerprinting

Repetitive extragenic palindromic polymerase chain reaction (rep-PCR) fingerprinting was performed for isolates from *Trifolium arvense, T. campestre, T. dubium, T. pratense* and *T. striatum*. The enterobacterial repetitive intergenic consensus (ERIC) primers (Table N.3, Appendix N) were chosen for their ability to distinguish bacteria at subspecies or strain level (Versalovic *et al.* 1994). Each 25 µl PCR reaction contained: 16.7 µl DNA-free water, 2.5 µl 10x PCR buffer, 1.6 µl dNTPs (concentration of 5 mM/µl), 1 µl of each primer (concentration of 25 µM/µl), 0.2 µl Taq polymerase (5 U/µl, HotStarTaq Plus, Qiagen) and 2 µl of template DNA. Amplifications were performed in a C1000 Touch™ Thermal Cycler (Bio-Rad) with the following cycle conditions: 94° C for 3 min followed by 35 cycles of 94° C for 1 min, 52° C for 1 min, 72° C for 1 min, with a final extension of 7 min at 72° C and held at 10° C . 8 µl of each PCR product was size-fractionated on 2% agarose gel in 1% TBE buffer at 100 volts for 2 h. At least one size standard (Generuler™ 1kb Plus; Thermo Scientific) was used per 15 wells. Gels were stained in ethidium bromide on a rotary shaker for 30 min and images were obtained using an Isogen ProXima 10Phi gel image system.

Quantitative fingerprints of DNA fragment sizes present in each sample were obtained from ERIC-PCR gel images using the software, GelAnalyzer 2010a (Lazar 2010), by sizing fragments relative to the known molecular weights of the DNA size standard (ladder). The sized molecular weights were log-transformed and complete linkage hierarchical clustering was implemented in R (R Development Core Team 2013) to: (a) assign DNA fragments with identical molecular weights in different samples into the same cluster; and (b) assign fragments with a distance less than 0.05 into the same cluster (R code based on Ishii, Kadota & Senoo 2009). The resulting data was converted into a presence-absence matrix of fragments in each sample. Non-metric multidimensional scaling (NMDS) ordination was performed to visualise the degree of similarity of fingerprints as a function of range (NZ/UK) and *Trifolium* host species (two dimensions, Jaccard distance measure and a maximum of 10,000 iterations using metaMDS function in version 2.0-10 of R package vegan Oksanen *et al.* 2013). The ordination produced a low stress value (0.13) and a nonmetric R^2 value between ordination distance and observed dissimilarity (0.98) that indicated a good ordination representation in two dimensions. We assessed the multivariate homogeneity of dispersion among samples (mean distance to the centroid of samples) to test whether NZ fingerprints were more homogeneous than UK fingerprints. This was based on Raup-Crick distances for 10,000 simulations (using the functions raup-crick and betadisper of R package vegan Chase *et al.* 2011; Oksanen *et al.* 2013) and ANOVA was used to test for significant differences. Only isolates authenticated as rhizobia by successful amplification with rhizobia-specific nodD primers (see below) were used for rep-PCR analysis (110 isolates used for rep-PCR in total; Appendix O).

5.3.5 Phylogenetic analysis of nodD gene

We chose the symbiotic nodD gene for phylogenetic analysis because it can exhibit genetic heterogeneity among bio-varieties of *Rhizobium leguminosarum* associated with different legume genera and specifically among strains of *Rhizobium leguminosarum* biovar *trifolii* (Zézé, Mutch & Young 2001; Mutch & Young 2004; Yates *et al.* 2008). This protein-coding gene is involved in the rhizobia-plant signalling process that partly determines the host range of rhizobia by controlling the response of the bacteria to plant flavonoids (Spaink *et al.* 1987; Wang *et al.* 2012). A region internal to nodD was amplified using the primer pair Y5 and Y6 (Zézé *et al.* 2001; Table N.3, Appendix N), generating sequences around 850 base pairs in length. These primers are rhizobia-specific, so successful amplification was used to authenticate isolates as rhizobia. Each 25 µl PCR reaction contained: 18.7 µl DNA-free water, 2.5 µl 10x PCR buffer, 1 µl dNTP mix (concentration of 5 mM/µl), 0.8 µl of each primer (concentration of 10 µM/µl), 0.2 µl Taq polymerase (5 U/µl, HotStarTaq Plus, Qiagen) and 1 µl of template DNA. Amplifications were performed in a C1000 Touch™ Thermal Cycler (Bio-Rad) with the following cycle conditions: 95° C for 2 min followed by 25 cycles of 95° C for 1 min,

59° C for 1 min, 72° C for 2 min, with a final extension of 5 min at 72° C and held at 10° C . Amplified products were purified and sequenced in both directions (EZ-Seq, Macrogen Europe).

Forward and reverse sequences were checked for quality, base ambiguities coded and assembled using DNA Baser (Heracle BioSoft) and then aligned using the Muscle algorithm (Edgar 2004) in MEGA 6 (Tamura *et al.* 2013). Sequences from 131 rhizobia isolates were suitable for phylogenetic inferences (Appendix O), which were trimmed to an equal length of 662 base pairs. A phylogenetic tree was constructed in MEGA 6 using maximum likelihood inference with 1000 bootstrap replicates, five discrete gamma categories and a gamma parameter of 0.31 using the T92+G model (Tamura 3-parameter model; (Tamura 1992), selected according to a model test in MEGA 6). The overall mean genetic distance among sequences (the average number of base substitutions per site, excluding ambiguous positions) was also calculated in MEGA 6 using the same parameters as the phylogeny. Four additional nodD rhizobia sequences were included in the phylogeny from Genbank: two from *Trifolium polymorphum* from Uruguay (strains WSM2302 and WSM2304), one from an unidentified annual *Trifolium* species in Greece (WSM1325), and one from naturalised *T. subterraneum* in Australia but with likely European origins (WU95) (Yates *et al.* 2008 and references therein; Genbank accessions: DQ873523.1, DQ873524.1, EU290605.1, EU290606.1). Further nodD sequences from clover rhizobia of UK provenance were available in Genbank (from Zézé *et al.* 2001; accessions: AJ306476.1, AJ306479.1 AJ306478.1 AJ306477.1) and were included in exploratory phylogenies. These sequences were not genetically distinct from our isolates and were thus not included in the phylogeny presented, given our existing sampling of clover rhizobia from the UK.

To test for a phylogenetic signal from the geographic range or *Trifolium* host that isolates originated from, we calculated maximum likelihood estimates of Pagel's lambda (Pagel 1994, 1999) using the fitdiscrete function in version 2.0.3 of the R package geiger (Harmon *et al.* 2008). Lambda varies from 0 to 1, where 0 corresponds to a complete absence of phylogenetic correlation and 1 indicates perfect correlation. To assess whether the phylogenetic signal of each variable was significant, likelihood ratio tests were performed comparing the value of λ from a tree transformed to have zero phylogenetic signal (λ = 0) to that of our tree topology. The likelihood ratio test followed a chi-squared distribution, with 1 degree of freedom.

5.3.6 16S sequencing

In order to identify the taxonomic identity of isolations from root nodules that failed to amplify with the rhizobia-specific nodD primers, sequencing of the 16S gene was performed using the universal bacterial primers 27f and 1492r (Lane 1991; Table N.3, Appendix N). Each 25 µl reaction contained: 18.3 µl DNA-free water, 2.5 µl 10x PCR buffer, 2 µl dNTPs (concentration of 5 mM/µl), 0.5 µl of each primer (concentration of 10 µM/µl), 0.2 µl Taq polymerase (5 U/µl, HotStarTaq Plus, Qiagen) and 1 µl

of template DNA. Amplifications were performed with the following settings: 95° C for 5 min followed by 35 cycles of 95° C for 30 s, 57° C for 40 s, 72° C for 1 m, with a final extension of 10 min at 72° C and held at 10° C. Amplified products were purified and sequenced in the forward direction (EZ-Seq, Macrogen Europe). Nucleotide sequences were checked for quality and trimmed using DNA Baser (Heracle BioSoft). BLASTn searches (Altschul *et al.* 1997) were performed to match isolations with sequences of known taxa in GenBank.

5.3.7 Inoculation experiment

To compare the nodulation ability and plant growth effects of root nodule bacteria from NZ and the UK, we conducted a glasshouse experiment in which we grew each *Trifolium* species in the presence of bacterial strains isolated from the same species in each range. Bacterial isolates from each *Trifolium* host species from each range were bulked to form glasshouse inoculum (7 species x 2 ranges = 14 inocula). To standardise the growth stage of isolates forming each inoculum, isolates were streaked onto YMA plates from glycerol stocks at the same time, cultured at 20° C for 2 days, then grown in 5 ml YMB solution on a rotary shaker at 28° C and 120 rpm for 48 h. The resulting liquid cultures were centrifuged at 5500 rpm, the supernatant removed and the remaining bacterial pellets re-suspended in 2 ml of sterile phosphate-peptone buffer solution (Table N.4, Appendix N). The respective 2 ml cell suspensions originating from each species in each range were then mixed to produce bulk inocula. The number of isolates used per inocula ranged between 13-15 because bacterial isolations from some root nodules were not successful; however, the number of isolates comprising the UK and NZ inocula for each clover species were kept equal. To ensure an approximately equal bacterial cell density in the NZ and UK inocula per species, the optical density (OD) of each bulk inoculum was measured using a spectrophotometer and dilutions were performed with sterile phosphate-peptone buffer to equalise the OD within species. The OD at 600 nm of each inocula ranged between 1-1.2 depending on *Trifolium* species, which represented 10^9 colony-forming units per ml, as verified by serial dilution and colony counts.

Seedlings were pre-germinated in sterile conditions in an incubator at 12° C with 8 h light per day for 14-16 days. Seeds were surface-sterilised for 2 min in a solution of 10% household sodium hypochlorite with a drop of Tween 20 surfactant and rinsed thoroughly with de-ionised water. Seeds were then hand scarified with a scalpel and sown in sterilised glass beads. Seeds from NZ and the UK were included to test whether plants are adapted to rhizobia from the same provenance. To avoid the possible confounding effect of local scale plant-rhizobia adaptation, seeds were sourced from field populations away from rhizobia sampling sites (Appendix M). Equal-sized seedlings were transplanted into 1 L pots of sterilised sandy-loam background soil (gamma-irradiated at > 25 KGray, Ede Isotron, The Netherlands) collected from a natural grassland (Planken Wambuis, Ede, The

Netherlands). The soil at the base of each seedling was then inoculated with 1 ml of inoculant. Ten plants of each seed provenance (UK/NZ) were grown with bacteria from each provenance (UK/NZ), for a total of 280 plants: 7 species x 2 seed ranges x 2 bacteria ranges x 10 replicates. Plants were grown in a glasshouse at a day/night temperature of 21° C /16° C and light supplemented to a 16 h day length. Pots were watered to an equal weight every 10 days and harvested after 39-46 days depending on species. Above- and belowground biomass was oven dried at 70° C for at least 48 h and then weighed. Before being disposed, all glasshouse soil washings and plant pots were sterilised by two rounds of autoclaving to 120° C for 20 min, with 24 h in between the rounds.

To evaluate the degree of effective nitrogen-fixation by rhizobia, root nodulation of each plant was scored at the time of harvest on a 0-3 scale adapted from Thrall et al. (2007) and Corbin et al. (1977), based on the size, number, position and colour of nodules. Functional nitrogen-fixing nodules are pink due to the presence of the pigmented-protein leghaemoglobin, whereas ineffective nodules are white (Somasegaran & Hoben 1994). Nodule position on the root system indicates the plant growth-stage at which rhizobia infection occurred. A score of zero represented no nodulation or white (ineffective) nodules only. Scores of 1-3 represented increasing degrees of effective N-fixation: 1 for a low number of small (< 1mm) pink nodules predominantly on lower roots; 2 for an intermediate number of larger pink nodules (many >1mm), with some at the root crown (top 2 cm of the root system); and 3 for abundant large (many > 1mm) pink nodules, particularly at the root crown.

To test for significant differences between the plant growth effects of rhizobia from NZ and the UK and for adaptation of *Trifolium* to rhizobia of the same range, linear regression models were fitted for each *Trifolium* species using log-transformed growth rates (dry-weight biomass per glasshouse-grown day) as the response variable, with rhizobia and seed range along with their interaction as fixed effects. We also fitted a linear model using data from all *Trifolium* species to test for the overall effect of root nodulation on plant growth, for which log-transformed growth rate was used as the response variable; rhizobia, seed range and their interaction as a fixed effect; nodulation score as a fixed effect; and species as a random effect. To compare nodulation ability, mean nodulation scores and 95% confidence intervals were calculated for each rhizobia range per species and relationships between nodulation score and plant growth rates were explored. Data was analysed in R version 3.0.1 (R Development Core Team 2013) and models fitted using version 1.7-03 of the R package arm (Gelman & Su 2014).

5.4 Results

All plants formed effective nitrogen-fixing nodules and nodulation score was a significant overall positive linear predictor of plant growth rate (slope = 0.08; df = 270; p = 0.001; Table P.1, Appendix P). Examining these nodulation x growth rate relationships separately by rhizobia and seed range, however, showed that there was not a significant positive relationship for NZ seed inoculated with NZ rhizobia (Appendix Q). Nodulation scores averaged over all *Trifolium* species were not significantly different between NZ and UK rhizobia (Figure 5.1). Furthermore, mean nodulation scores were generally not significantly different between the ranges for each *Trifolium* species (Figure 5.2). There were also no significant differences in plant growth rate in response to NZ and UK rhizobia for each *Trifolium* species (Figure 5.3; Table P.2, Appendix P). In contrast to expectations of regional plant-rhizobia adaptation, there were no indications of plants benefitting more from rhizobia isolated from the same range (Figure 5.2, Figure 5.3), as confirmed by the lack of significant interaction between rhizobia and seed range per *Trifolium* species (Table P.2, Appendix P).

Molecular identification of the bacterial isolates comprising each rhizobia inocula in the glasshouse experiment unfortunately revealed that there were a number of non-rhizobia taxa present in the inocula, although each inoculum consisted of at least 53% rhizobia strains (Appendix R). GenBank BLAST searches with the 16S rRNA sequences from the non-rhizobia isolates indicated matches to a range of bacterial taxa (Appendix S), some potential plant pathogens and other potential plant growth-promoting bacteria. Six 16S sequences were not good enough quality for identification (isolate references: s31u, o52n, r22n, o13n, d11n, c12n).

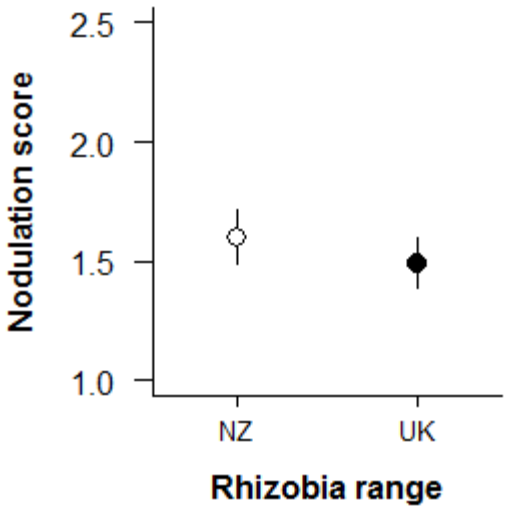

Figure 5.1. Mean nodulation score averaged over all *Trifolium* species for rhizobia from the introduced (NZ) and native range (UK). Bars represent 95% confidence intervals.

The rep-PCR analysis of rhizobia-authenticated isolates indicated a high overall richness of rhizobia strains, as 97 out of the total 110 fingerprints were unique. In some cases, the three isolates from the same *Trifolium* species from the same field site produced different fingerprints. Ordination of the fingerprint data did not suggest any separation based on the rhizobia range or *Trifolium* host (Figure 5.4). Multivariate dispersion of the strain fingerprints was not significantly different between the ranges (0.19 NZ, 0.19 UK; n = 110, p = 0.65), indicating that the richness of fingerprints was equally high in NZ and UK.

The nodD sequences from isolates in this study were reasonably conserved, with a low overall mean genetic distance of 0.06. Many nodes on the phylogenetic tree (Figure 5.5) were not well supported due to this low genetic variability. Nonetheless, the phylogeny illustrates that isolates from NZ had very similar sequences to those from the UK, and there were no phylogenetic groupings based on rhizobia range. Indeed, many groups on the phylogeny consisted of isolates from a mixture of *Trifolium* host species from both ranges. The phylogenetic separation of rhizobia from annual and perennial *Trifolium* species was generally also limited, although there was one group of isolates found only in annuals. The lack of phylogenetic signal based on isolate range (NZ/UK) or *Trifolium* host was confirmed by Pagel's lambda ($\lambda = 0$ and $p = 1.0$ for both variables).

There was high sequence divergence between our isolates and two Genbank rhizobia sequences from Uruguayan *T. polymorphum*, which formed the root of the tree. The other two GenBank rhizobia sequences originating from *T. subterraneum* in Australia (probably of European origin) and an unidentified annual *Trifolium* species in Greece resolved within our samples.

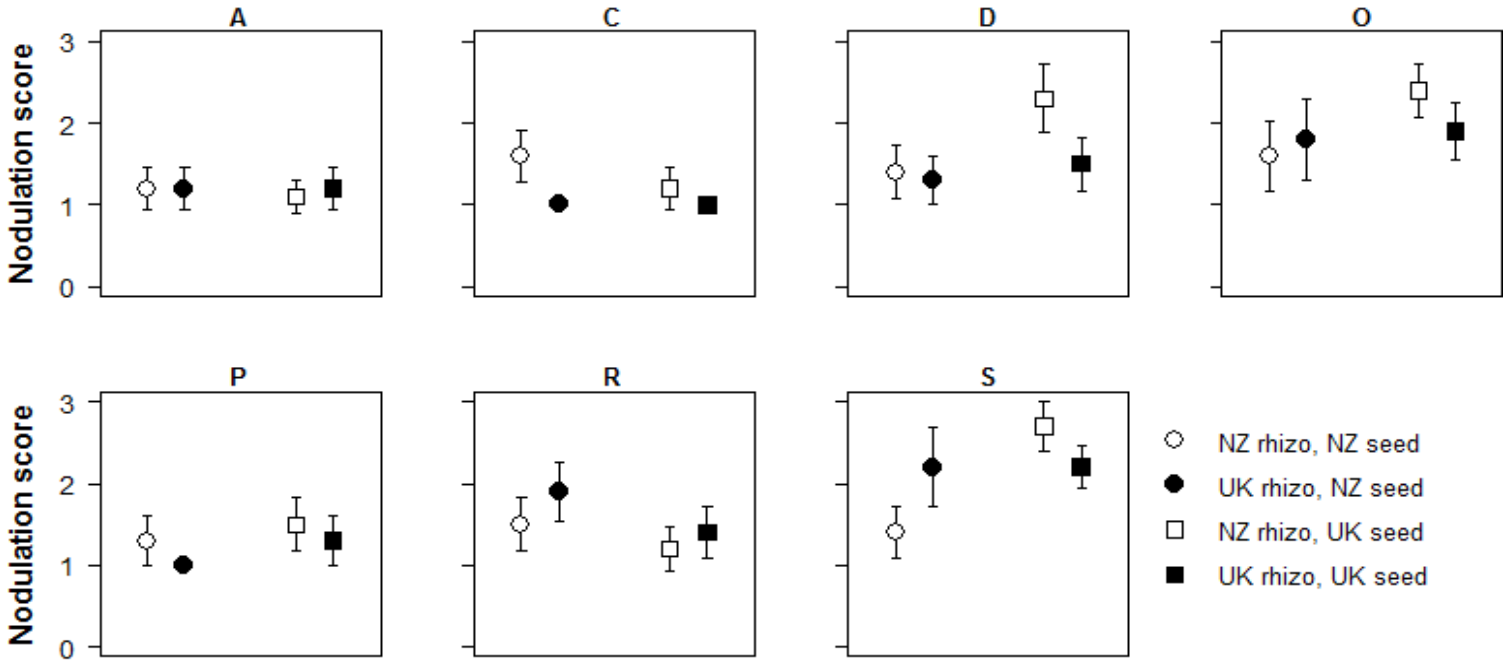

Figure 5.2. Mean nodulation scores for each *Trifolium* species by rhizobia range (NZ = white symbols; UK = black symbols) and seed range (NZ = circles; UK = squares). Plot titles are *Trifolium* species abbreviations: A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum*. Bars represent 95% confidence intervals.

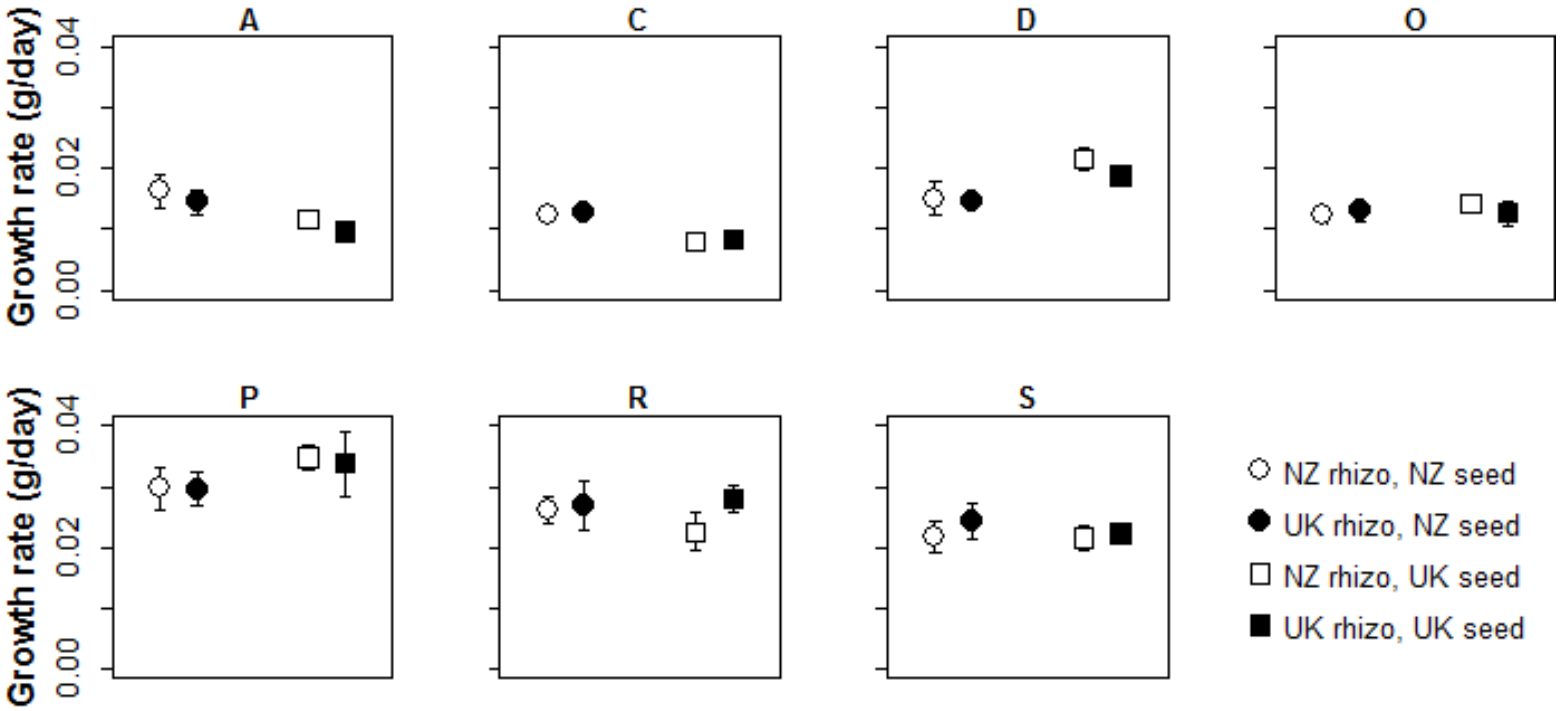

Figure 5.3. Mean plant growth rate (grams of dry weight biomass per glasshouse-grown day) for each *Trifolium* species by rhizobia range (NZ = white symbols; UK = black symbols) and seed range (NZ = circles; UK = squares). Plot titles are *Trifolium* species abbreviations: A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum.* Bars represent 95% confidence intervals.

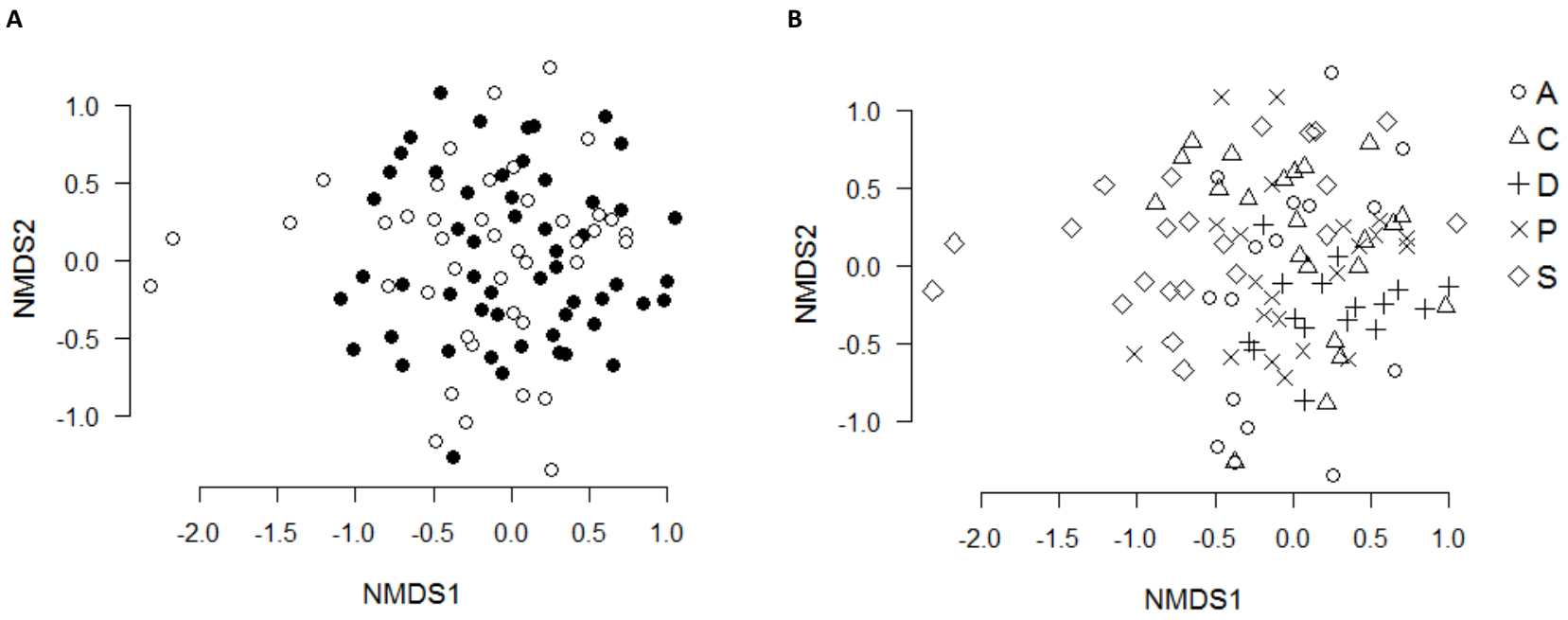

Figure 5.4. NMDS ordinations of rhizobia isolate ERIC-PCR fingerprints according to A) geographic range (NZ = ○, UK = ●) and B) *Trifolium* host (key indicates *Trifolium* species: A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* P = *T. pratense,* S = *T. striatum*).

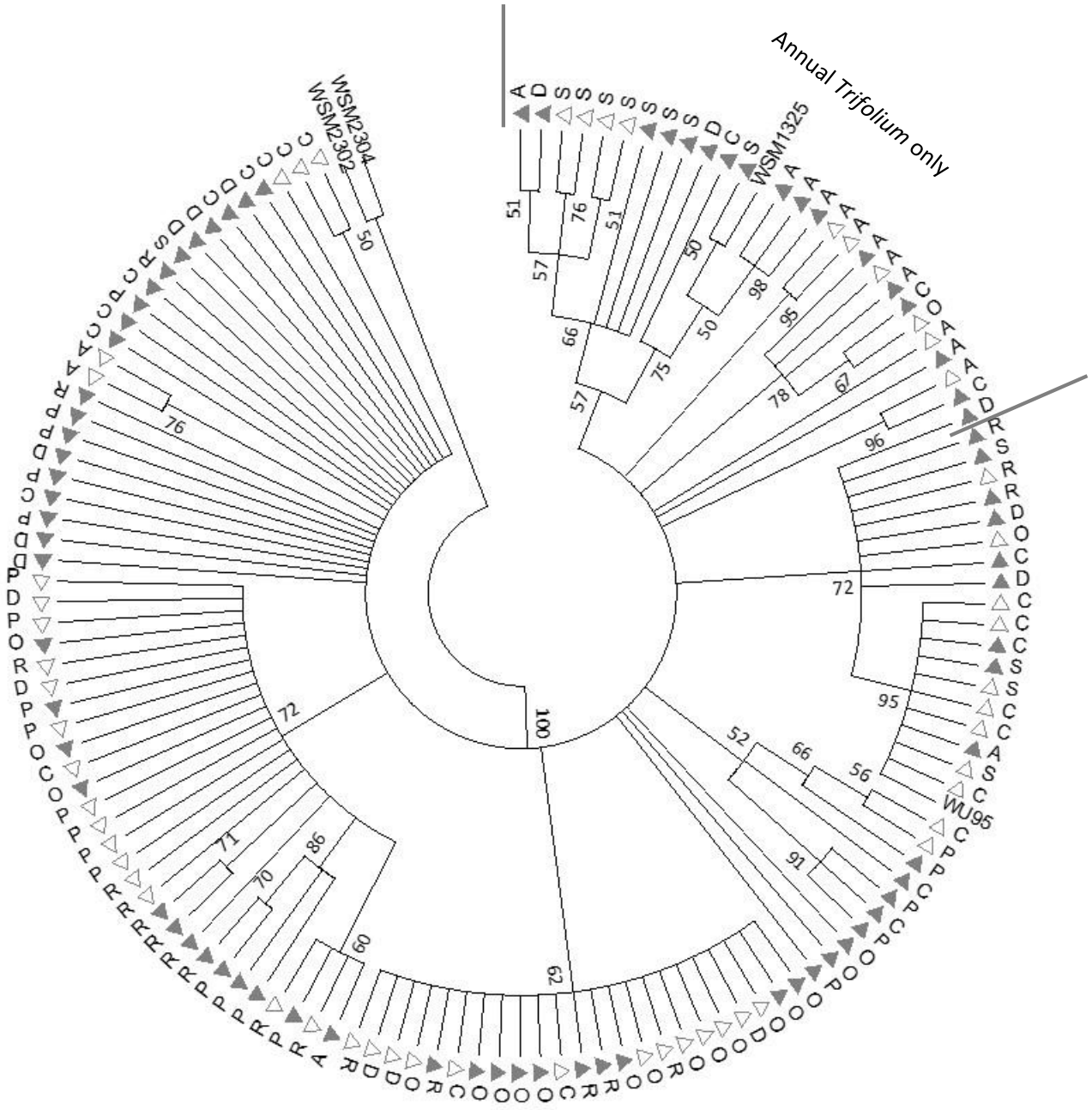

Figure 5.5. Phylogeny of 135 nodD sequences from clover-associated rhizobia strains originating from NZ (△) and the UK (▲). Tip labels indicate *Trifolium* host species: A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum*. Tip labels WSM2304, WSM2302, WSM1325 and WU95 represent sequences from Genbank originating from: two *T. polymorphum* from Uruguay, *T. subterraneum* from Australia and an annual *Trifolium* species from Greece. Analysis was performed using Maximum Likelihood in MEGA 6 (Tamura *et al.* 2013) with the Tamura 3-parameter model with five discrete gamma categories and a gamma parameter of 0.31. Percentage bootstrap support is shown for nodes where this exceeds 50%, and nodes with bootstrap support lower than 50% were collapsed into polytomies.

5.5 Discussion

Although a number of studies have investigated how rhizobia affect legume invasion by making biogeographical comparisons between plant's introduced and native ranges (Lafay & Burdon 2006; Parker *et al.* 2007; Rodríguez-Echeverría *et al.* 2011; Birnbaum *et al.* 2012; Ndlovu *et al.* 2013; Crisóstomo *et al.* 2013), few have examined the strain richness, genetic relatedness and the symbiotic effectiveness of rhizobia in both ranges (but see Callaway *et al.* 2011; Birnbaum *et al.* 2012). Furthermore, the majority of these studies examined legume species that are promiscuous in their rhizobia associations which may be less constrained by access to rhizobia in their introduced ranges. We hypothesised that NZ-naturalised annual *Trifolium* – most of which were accidentally introduced – would be limited by an absence of optimal coevolved rhizobia strains, while expecting agricultural perennial *Trifolium* to have access to highly mutualistic strains in NZ. We also expected populations of clover-associated rhizobia to exhibit lower strain richness in NZ relative to the native European range. In contrast, however, our results indicate that all seven *Trifolium* study species have access to a high richness of effective nitrogen-fixing rhizobia strains in NZ that have likely facilitated their successful naturalisation.

Studies using DNA fingerprinting have previously indicated that introduced populations of clover rhizobia can exhibit high richness (Granada *et al.* 2013), including in soils within established clover pastures in Australia and NZ (Demezas *et al.* 1995; Nangul *et al.* 2013). However, it was not known whether this richness was comparable to coevolved rhizobia populations in the native range, particularly for naturalised non-agricultural *Trifolium* species. The molecular analyses in this study indicate that naturalised clover-associated rhizobia in NZ soils are genetically diverse, with a comparable strain richness to in the European native range. This argues against the occurrence of a genetic bottleneck caused by small founder populations of rhizobia, as appears to be the case for introduced rhizobia populations associated with other alien legume species, such as *Trifolium ambiguum*, *Lens culinaris* (lentil), *Pisum sativum* (pea) and *Phaseolus vulgaris* (bean), in different regions (Seguin *et al.* 2001; Riah *et al.* 2014; Junier *et al.* 2014).

The nodD phylogenetic analyses did not suggest any genetic separation of NZ and UK rhizobia. In order to fully establish the origin of clover rhizobia in NZ, we would need to examine the phylogenetic relationships between our isolates and those from other countries using multiple gene regions. Nevertheless, rhizobia associated with *Trifolium* species native to other centres of diversity (Africa and America) are generally incompatible with most European *Trifolium* (Burton 1985 and refs. therein), suggesting that it is highly likely that rhizobia populations associated with these European *Trifolium* species in NZ are coevolved strains that have been introduced from Europe. The

extensive importation of biological material to NZ by European settlers from the mid-19th century (Allen & Lee 2006) probably resulted in multiple inadvertent introductions of clover rhizobia to NZ, possibly from more than one European region. Indeed, a variety of plants with intact root systems were imported to NZ from the UK (e.g., Herriott 1919), providing a possible vector for rhizobia transport. Other materials such as introduced plant seeds may also have harboured rhizobia (e.g., Pérez-Ramírez *et al.* 1998). Such inadvertent introduction of rhizobia, combined with the widespread use of agricultural inoculants, probably explains the existence of these rich populations of clover rhizobia in NZ.

Although the phylogenetic grouping of rhizobia comprised of isolates originating solely from annual *Trifolium* was not well supported by bootstrap values (Figure 5.5), this grouping may represent rhizobia strains that are specific to the annual species, in accordance with the findings of a cross-inoculation study showing that perennial *Trifolium* were often incompatible with rhizobia from annuals (Howieson *et al.* 2005). This grouping nonetheless included rhizobia isolations from NZ and thus, if these strains are annual-specific, they are available in NZ. Actual cross-inoculation tests would, however, be needed to elucidate whether they are annual-specific strains. Considering that the nodD gene is partly responsible for determining the host range of rhizobia (Spaink *et al.* 1987; Wang *et al.* 2012) and that much of the nodD phylogeny did not differentiate between rhizobia from annual and perennial hosts, the rhizobia intentionally introduced for perennial *Trifolium* may in fact effectively nodulate and facilitate the naturalisation of the annual species studied here. Alternatively, it is possible that rhizobia strains intentionally introduced for the agricultural annual species *T. subterraneum* have facilitated the spread of other European annual *Trifolium* species.

The findings from the glasshouse experiment indicated that plants receive similar symbiotic benefits from NZ and UK rhizobia. It is, however, difficult to make solid conclusions about the plant growth effects based on this data, given the presence of non-rhizobia bacterial taxa in many of the inocula that probably contributed to the plant growth responses (Appendix R). The isolates from NZ nodules comprised of higher number of non-rhizobia taxa than the UK isolates (Appendix S), however, this may be an artifact of a dry NZ summer at the time of sampling, causing nodules to begin senescence and allow them to be colonised by a more diverse community of bacterial taxa. Nevertheless, these non-rhizobia taxa would not have influenced the nodulation score variable that assessed the degree of effective nitrogen-fixation. The similarity in nodulation scores between the rhizobia ranges (Figure 5.1, Figure 5.2) indicates that the seven *Trifolium* study species have access to nitrogen-fixing rhizobia in NZ that are as symbiotically effective as in the European native range. The differing geographic success of these *Trifolium* species in NZ therefore does not appear to have been

influenced by rhizobia limitations. Our findings suggest that the populations of clover rhizobia that are now widespread in NZ soils (Lowther & Kerr 2011) are not only suitable for agricultural *Trifolium* species, but also for many non-commercial *Trifolium* species. Low rhizobia densities may, however, still limit the study species in NZ soils that lack naturalised clover, such as land cleared from virgin native scrub or forest.

Given that the study species are all native to Western Europe and that *Trifolium* from other provenances have been shown to differ in their rhizobia requirements (e.g., Tesfaye & Holl 1999), we do not suggest that compatible rhizobia are available for all *Trifolium* species in NZ. There is, for example, evidence that naturalised rhizobia in NZ are limiting to the growth of potential new agricultural *Trifolium*, such as the Eastern European species *T. ambiguum* (Elliot & McIntyre 1998; Pryor *et al.* 2004). The ongoing introduction of new clover rhizobia strains to NZ, such as strains selected for high symbiotic ability with new agricultural clover species may, over time, change the rhizobia relationships of other *Trifolium* species in NZ. Rhizobia strains suitable for *T. ambiguum*, for example, form parasitic nodules on the pasture species *T. repens,* and can even reduce the performance of *T. repens* when present at high densities (Elliot & McIntyre 1998; Pryor *et al.* 2004).

Our results did not support expectations of regional plant-bacterial adaptation, as *Trifolium* genotypes did not perform better with rhizobia from the same provenance. It is possible that plant-bacterial adaptation operates at more of a local scale under the influence of common selection pressures on both the plant and bacterial populations from site-specific abiotic conditions. Clover-rhizobia adaptation has indeed been documented more at the field site scale (e.g., Sherwood & Masterson 1974; Mytton 1975), and specificity in plant-bacterial associations may therefore still exist in our system at a more local level.

Overall, our results support the findings of many other studies showing that naturalised legumes are often co-introduced with their coevolved rhizobia mutualists (Stepkowski *et al.* 2005; Wei *et al.* 2009; Andrus *et al.* 2012; Ndlovu *et al.* 2013; Crisóstomo *et al.* 2013). This can represent a co-invasion scenario, in which both alien partners mutually facilitate each other's persistence and spread (Nuñez & Dickie 2013). In some cases, rhizobia invaders can even disrupt native symbioses by forming novel associations with native plants, but provide them with inferior benefits relative to native rhizobia (Rodríguez-Echeverría 2010; Rodríguez-Echeverría *et al.* 2012). Such disruption of native symbioses is more likely to involve plant species and bacteria strains that are capable of associating with a wide range of symbiotic partners, given that this promiscuity provides a higher chance for the formation of novel associations. However, Weir *et al.* (2004) detected that native NZ legumes were capable of associating with the more host-specific introduced rhizobia species

Rhizobium leguminosarum, despite native legumes predominantly associating with native *Mesorhizobium*. There are several biovarieties of *Rhizobium leguminosarum* that are characterised by their restricted plant host ranges, such as within the genera *Trifolium*, *Vicia* and *Phaseolus*, so the association of this rhizobia species with NZ native plants likely represents a novel association. This host-broadening of rhizobia may have arisen due to horizontal gene transfer (Freiberg *et al.* 1997; Ochman, Lawrence & Groisman 2000) that has allowed introduced rhizobia strains to acquire novel genes from native NZ rhizobia species (Weir *et al.* 2004). Horn *et al.* (2014), for example, indicated that horizontal gene transfer has allowed native North American rhizobia to nodulate the alien plant *Cytisus scoparius* by acquiring symbiotic genes from introduced Spanish rhizobia. Indeed, such rapid evolution is widespread and common in bacteria, as genetic recombination can generate new genotypes (Andrade-Domínguez *et al.* 2014). Although further research is required, these examples suggest that rhizobia relationships should be viewed as dynamic, as introduced rhizobia may diversify post-establishment.

Chapter 6: General discussion

6.1 Thesis aims

The overall aim of this study was to investigate how soil biota influences the plant invasion process. Interactions between *Trifolium* species and their associated soil biota were examined in the introduced range of New Zealand and the native range of Europe. Such biogeographical contrasts are key to explaining why the performance of some plant species is improved in the introduced range (Hierro *et al.* 2005). The following key questions were tested with the NZ-naturalised *Trifolium* study system: a) Do *Trifolium* escape inhibitory plant-soil feedback (PSF) in NZ?; b) Is the escape from inhibitory PSF transient, i.e. do longer-naturalised and more widespread species in NZ experience a weaker strength of escape?; c) Do *Trifolium* associate with a lower richness of arbuscular mycorrhizal fungi in NZ relative to in the native range?; and d) Do NZ-naturalised *Trifolium* species, including annuals, have access to communities of nitrogen-fixing rhizobia bacteria that are as rich and symbiotically effective as in the native range?

6.2 Key results

6.2.1 Do *Trifolium* escape inhibitory PSF in NZ?

In Chapter 3, I firstly identified whether NZ-naturalised populations of 11 *Trifolium* species escape the inhibitory effects of soil biota by comparing PSF responses between NZ and Europe. Such cross-continental tests of whether alien plants are released from inhibitory PSF relative to their native range are uncommon. The novel statistical approach outlined in Chapter 2 was used to facilitate this comparison by producing estimates of PSF responses as if rhizobia bacteria were absent from the soil.

The strength of release from inhibitory PSF was highly specific to each *Trifolium* species, and this degree of among-species variation may be surprising, given the fine taxonomic scale of the study system. Although previous studies have indicated that the degree of biogeographic escape from inhibitory PSF varies among alien plant species: some benefitting (e.g., Reinhart *et al.* 2003; Callaway *et al.* 2011; Maron *et al.* 2014), while others not (e.g., Beckstead & Parker 2003; Maron *et al.* 2014); my findings indicate that the strength of escape can be highly variable among congeneric plant species in the same geographic region. This highlights that the strength of escape from inhibitory PSF is not readily generalisable across species, no matter how closely related they are.

The majority of studies testing the hypothesis of escape from inhibitory PSF have focussed on problematic invasive plant species which, given their success, may be a subset of alien species that are most likely to have benefitted from escaping inhibitory PSF. As a consequence, the degree to which less successful alien plant species experience enemy release is not well understood (Colautti *et al.* 2004). The less geographically widespread NZ-naturalised *Trifolium* species included in this study may be considered a form of negative control, illustrating that escaping inhibitory PSF is not only confined to highly successful plant invaders. Escaping inhibitory PSF is indeed unlikely to be the sole driver of invasive success, and a wide range of other factors, such as human-mediated propagule pressure, or the physiological attributes of the plant species can also be important determinants of invasion outcomes.

Examining the biogeographic differences in PSF for each *Trifolium* species individually showed that only two out of the 11 species experienced a significant release from inhibitory PSF in NZ. The lack of significant release experienced by many of the *Trifolium* species may be a result of soil-borne enemies having caught up to NZ from the European native range or the acquisition of novel soil-borne enemies in NZ. The biotic constraints on *Trifolium* productivity caused by plant antagonists in NZ are well documented in an agricultural context (e.g., Woodfield *et al.* 1996). Native generalist herbivores can be a significant source of biotic resistance to plant invasion (Maron & Vilà 2001; Parker & Hay 2005) and evidence from agricultural literature suggests that native soil-borne herbivores do impose control on *Trifolium* populations in NZ. The larvae of the endemic grass grub (*Costelytra zealandica;* a scarab beetle) feed on clover roots and are a major pest of clover-based pasture in NZ (East & King 1977), for example. The endemic mānuka scarab beetles (*Pyronota* spp.) are a second example of native NZ herbivores broadening their host range to attack *Trifolium* (Townsend *et al.* 2010; Zydenbos *et al.* 2011).

There are also examples of *Trifolium* enemies being accidentally introduced to NZ from Europe: larvae of a species of European clover root weevil (*Sitona lepidus*), for example, severely impacts *Trifolium* growth in NZ by consuming roots and root nodules (Barratt *et al.* 1996; Crush & Ouyang 2007; Lane 2011). Moreover, introduced enemies such as *Sitona lepidus* may be particularly damaging to clover growth if they themselves benefit from escaping their own enemies (although one biocontrol agent has since been introduced to NZ to supress *Sitona lepidus* populations (Gerard, Wilson & Eden 2011)). Plant-parasitic root knot (*Meloidogyne trifoliophila* and *M. hapla*) and cyst (*Heterodera trifolii*) nematodes are also abundant in NZ soils (Skipp & Christensen 1983; Mercer & Miller 1997; Mercer *et al.* 2008; Zydenbos *et al.* 2011), in addition to many soil-borne microbial pathogens of *Trifolium* (Skipp & Christensen 1983; Skipp *et al.* 1986). Chemical treatment to remove

nematodes from NZ pasture soils has been shown to result in a 40% increase in clover yield and 55% increase in nitrogen fixation (Watson *et al.* 1985). Differences in the susceptibility or tolerance of the *Trifolium* study species to these enemies may explain the high degree of among-species variation in the escape from inhibitory PSF. The larvae of legume weevils (*Sitona* spp.), for instance, preferentially feed on certain *Trifolium* species (Murray & Clements 1994).

6.2.2 Is the escape from inhibitory plant-soil feedback transient?

The greatest strength of the *Trifolium* study system was the availability of a group of species varying in their naturalisation dates and geographic spread that could be used to examine whether the escape from inhibitory PSF was transient. While studies have indicated that the richness of enemy species accumulates with longer introduction and larger geographic spread of alien plant hosts (e.g., Mitchell *et al.* 2010), few have tested for a transient enemy escape in context of inhibitory soil biota. A handful of studies have shown that alien plants accumulate inhibitory PSF within their introduced range (Nijjer *et al.* 2007; Diez *et al.* 2010; Dostál *et al.* 2013), but it is unclear whether the plant species used in these studies actually benefit from escaping inhibitory PSF relative to their native range. In addition, Dostál *et al.* (2013) and Nijjer *et al.* (2007) were based on single plant species and were unable to look for a correlation between the strength of PSF and the geographic spread of the alien plant populations. The present study appears to be the first to test whether the biogeographic escape from inhibitory PSF (i.e. the difference in PSF between the introduced and native range) is temporary.

The hypothesis that the strength of escape from inhibitory PSF would decline with longer time since naturalisation of the species was not supported; in fact, the longest-naturalised species (*T. micranthum*) experienced the strongest escape. It is therefore unclear whether a transient escape from inhibitory PSF is a generalisable phenomenon, given the low number of studies supporting this. The accumulation of inhibitory PSF possibly acts on more of a local than on a regional scale, where field sites occupied by a plant species for longer accumulate a higher richness or density of antagonistic soil biota that drive increasingly more negative PSF and limit the persistence of that plant species (as indicated by Nijjer *et al.* 2007 and Dostál *et al.* 2013). Indeed, this local build-up of soil-borne enemies is the motivation behind the widely recognised practice of crop rotation (Shipton 1977; Olsson & Gerhardson 1992) and is the main mechanism by which PSF can encourage the successional replacement of plant species (Kardol *et al.* 2013).

While the geographic spread of the *Trifolium* species was also not an overall predictor of the strength of escape from inhibitory PSF, averaging the strength of escape over species showed that

less widespread, mostly accidentally introduced *Trifolium* experienced a significant escape, whereas more widespread, mostly agricultural species did not. This suggests that either the geographic spread or the agricultural status of species may underlie a degree of variation in the strength of escape from inhibitory PSF. Agricultural plant species have previously been shown to support a higher richness of aboveground pathogens (Clay 1995; Mitchell *et al.* 2010), but as in my study, these correlations were also influenced by the geographic spread of species. An additional factor which may influence the strength of escape from inhibitory PSF is a plant species' life history: whether annual or perennial. I was, however, unable to test for differences based on life history because this would have been confounded by agricultural status, as the species selection did not include any non-agricultural perennials.

The strength of escape from inhibitory PSF was similar among *Trifolium* species within the same taxonomic group, suggesting that the phylogenetic relatedness to coexisting alien species in new regions may influence the strength of escape. Although more closely related plant species have been shown to experience more similar PSF responses (Brandt *et al.* 2009; Diez *et al.* 2010), no other studies appear to have indicated this at such a fine taxonomic scale. This finding potentially suggests that future experiments investigating whether alien plants escape inhibitory PSF should control for the phylogenetic relatedness of alien plants to other alien plant species in recipient communities, but directs tests of this are required.

6.2.3 Have NZ-naturalised *Trifolium* retained soil-borne mutualists?

Differences in the availability of arbuscular mycorrhizal fungi (AMF) and rhizobia between NZ and Europe may have contributed to the PSF responses quantified in Chapter 3, so these components of the soil community were examined in greater detail. An absence of optimal rhizobia strains for annual *Trifolium* species in NZ may have reduced any benefits gained from escaping inhibitory soil biota and partly explain the lower geographic success of these species in NZ. The benefits of escaping inhibitory PSF may, alternatively, have made up for the loss of optimal rhizobia or AMF mutualists. For example, when Callaway *et al.* (2011) examined the effects of the entire soil community on the growth of *Robinia pseudoacacia,* introduced populations were shown to escape inhibitory PSF relative to the native range, despite receiving inferior benefits from AMF.

AMF

In Chapter 4, the richness and community structure of AMF associated with three clover species (*T. arvense*, *T. repens* and *T. fragiferum*) in the UK and NZ were examined to identify whether *Trifolium* associate with a lower richness of AMF taxa in NZ relative to the native range. It is widely assumed

that AMF are available to alien plant species (Pringle *et al.* 2009), but it appears that only one study has conducted a biogeographical comparison of the richness and identities of AMF associated with a plant species in both its introduced and native range (Moora *et al.* 2011). The results of Moora *et al.* (2011) showed that differences in AMF assemblages between the introduced and native range can occur. Such biogeographical differences in AMF associations have the potential to influence plant performance and the subsequent success of alien plant populations, suggesting that the potential role of AMF in facilitating or constraining plant invasion should not be overlooked.

The results in Chapter 4 showed that at least three *Trifolium* species have access to an equal richness of AMF taxa in the naturalised range of NZ as in the native range of UK. The taxonomic structure of the *Trifolium*-associated AMF communities also appeared to be similar in NZ and UK, which may have resulted from *Trifolium* being co-introduced to NZ with AMF harboured in soil or biological materials imported from Europe, or because there are cosmopolitan AMF taxa native to both the UK and NZ. Given the general low plant host-specificity of AMF taxa (Klironomos 2000), the availability of European AMF in NZ may have facilitated the naturalisation of many *Trifolium* species, in addition to wide range of European plant species that comprise a large proportion of the naturalised flora (Howell & Sawyer 2006). Direct DNA sequencing is, however, needed to determine whether the shared AMF taxa between the ranges are the same AMF species, given that the species-discriminatory power of TRFLP is poorer than other sequencing techniques (Kohout *et al.* 2014).

The between-range similarities in AMF richness and possibly AMF community structure may suggest that AMF potentially provide a similar degree of mutualism to *Trifolium* in the introduced and native ranges. However, given that AMF can parasitise plants (Johnson *et al.* 1997), explicit quantification of the AMF growth effects in each range would be required to ascertain whether mutualistic benefits are equal in each range. Even if AMF identities in each range are the same, functional between-range differences in AMF effectiveness may exist due to factors that interact with AMF effectiveness. The benefits that AMF provide to *Trifolium* in the field, for example, will depend on the identities of other plant species connected to the common mycorrhizal network, as some plants can parasitise the network (Carey *et al.* 2004). Interactions between *Trifolium* and pathogens in both ranges will also influence plant growth responses resulting from AMF infection, given that AMF can provide a degree of pathogen protection to plants (Whipps 2004; Sikes *et al.* 2009).

Rhizobia

Given that the effect of rhizobia was statistically removed from the PSF responses in Chapter 3, it was important to identify whether there were differences in the symbiotic effectiveness of rhizobia between NZ and Europe. In Chapter 5, I hypothesised that agricultural *Trifolium* species would

receive similar benefits from rhizobia in each range due to the widespread use of agricultural clover rhizobia inoculants in NZ, but that NZ-naturalised annual clover species may be limited by a lack of optimal rhizobia strains. Conversely, the results indicated that naturalised populations of all seven *Trifolium* species studied, including five annual species, have access to rhizobia strains that have a comparable symbiotic effectiveness as rhizobia from the native range (UK). The variable geographic success of the *Trifolium* species in NZ has therefore probably not been influenced by rhizobia limitations. The availability of suitable AMF, in addition to rhizobia, has likely facilitated the naturalisation of *Trifolium* in NZ, given that the effectiveness of the rhizobia symbiosis and resulting legume growth benefits are greatly enhanced in the presence of suitable AMF (Crush 1974; Chalk *et al.* 2006; Abd-Alla *et al.* 2014).

Given that soils in NZ lacked clover rhizobia before the arrival of European colonists (Greenwood 1976) and that the nodD phylogeny did not differentiate between clover rhizobia from NZ and the UK, this study provides support for the growing body of evidence suggesting that legume species are often co-introduced with coevolved rhizobia from their native range (e.g., Stepkowski *et al.* 2005; Wei *et al.* 2009; Crisóstomo, Rodríguez-Echeverría & Freitas 2013). The DNA fingerprinting additionally showed that naturalised rhizobia populations associated with alien legumes can exhibit high strain richness, comparable to the richness of rhizobia communities in the native range. This contrasts with other studies finding that naturalised rhizobia populations can have low genetic diversity, indicative of a genetic bottleneck (Seguin *et al.* 2001; Riah *et al.* 2014; Junier *et al.* 2014). The high richness of clover rhizobia strains in NZ may have resulted from the intentional introduction of multiple agricultural strains, combined with further inadvertent introductions, as well as potential post-introduction bacterial evolution.

As stated in Chapter 5, however, rhizobia strains do vary in their cross-compatibility and symbiotic effectiveness among *Trifolium* species, and a lack of effective rhizobia may, therefore, still limit other *Trifolium* species in NZ. Out of the 54 *Trifolium* species introduced to NZ, 29 failed to naturalise (Gravuer *et al.* 2008), and it is still possible that a lack of suitable rhizobia strains contributed to these failures. *T. ambiguum*, for example, associates with specific strains of *Rhizobium leguminosarum* biovar *trifolii*, and the commercialisation of this species in NZ would likely require widespread introduction of these strains (Elliot & McIntyre 1998; Pryor *et al.* 2004). Nevertheless, a number of other *Trifolium* species not included in the rhizobia analyses in Chapter 5 probably also have access to a rich community of rhizobia in NZ, given that many other *Trifolium* share the same rhizobia 'effectiveness group' (Burton 1985) as the species studied.

In addition, the nodD phylogeny generally did not distinguish between rhizobia associated with agricultural and non-agricultural *Trifolium* species. It is thus possible that the naturalisation success of *Trifolium* species accidentally introduced to NZ has been facilitated by the clover rhizobia strains widely introduced as agricultural inoculants. Given the similarity in AMF communities among the three *Trifolium* species indicated in Chapter 4, it is also possible that the accidentally introduced *Trifolium* species utilise the AMF communities that have developed in NZ soils in association with agricultural *Trifolium* species.

6.2.4 Synthesis of Chapters 3-5

The *Trifolium* species tested appear to encounter similar mutualistic interactions with soil biota in NZ and Europe and this has likely facilitated their successful naturalisation in NZ. While alien plant species used for agriculture are most likely to retain suitable mutualists via their intentional co-introduction, my results suggest that these agricultural plant species may be less likely to escape inhibitory soil biota. The *Trifolium* species that have been shown to experience a significant escape from inhibitory PSF in NZ, in addition to retaining their rhizobia and probably AMF mutualists, are not considered to be problematic invaders (e.g., *T. campestre*). Although escaping antagonistic soil biota and retaining mutualists is likely to have contributed to the successful naturalisation of these plant species, my findings suggest that these factors may not be important in determining whether alien species become inordinately successful to the extent that they are considered invasive.

6.3 Additional influences on alien plant success

The success of an alien plant species can be determined by an abundance of factors as well as their associated soil biota (Theoharides & Dukes 2007), such as the degree of human-mediated dispersal (Pyšek, Krivánek & Jarosík 2009; McGregor *et al.* 2012). In fact, Gravuer *et al.* (2008) identified that human factors have played a significant role in determining the relative success of NZ-naturalised *Trifolium* species. The results of Gravuer *et al.* (2008) highlighted the importance of propagule pressure, as the extent of intentional planting of *Trifolium* was identified as a strong determinant of the spread rate of intentionally introduced species in NZ. In addition, the frequency of occurring as contaminants in agricultural seed stocks appears to have strongly influenced the spread rate of accidentally introduced species. Out of the biogeographic attributes tested by Gravuer *et al.* (2008), *Trifolium* species with a large native range were more likely to attain large geographic spread in NZ. Biological traits played a weaker role in determining the naturalisation success of *Trifolium* in NZ, although longer-flowering species appeared to spread more rapidly. Most of the variables tested by Gravuer *et al.* (2008) did not involve biotic interactions, but the study did detect the importance of

rhizobia, as *Trifolium* species for which compatible rhizobia has apparently been introduced to NZ (based on rhizobia effectiveness groups) were more likely to naturalise.

Aboveground biotic interactions, such as pollination, can also influence the naturalisation success of alien plants, and the lack of suitable pollinators seemingly limited the early establishment of *Trifolium* in NZ (Hopkins 1914). Gravuer *et al.* (2008) also found some evidence for this, as self-pollinating *Trifolium* species apparently spread more rapidly in NZ. Pollination limitations to *Trifolium* in NZ were subsequently overcome by the introduction of generalist European bee species (Godley 1979). The lower geographic success of some NZ-naturalised *Trifolium* species may additionally be a result of poorer competitive ability, given that the less widespread species are more successful in drier habitats (e.g., Boswell *et al.* 2003), where interspecific competition is often less intense.

The *Trifolium* species may also differ in the degree to which they have escaped aboveground enemy regulation, as this is can be highly variable among plant species (e.g., Mitchell & Power 2003; Agrawal *et al.* 2005). Many aboveground enemy taxa of *Trifolium* species are prevalent in NZ, including numerous foliar viruses, such as white clover and alfalfa mosaic virus (Guy 2014). Aboveground clover pests accidentally introduced to NZ include the European cover flea (a species of springtail; *Sminthurus viridis*), European stem nematode (*Ditylenchus dipsaci*) and Australian field cricket (*Teleogryllus commodus*) (Woodfield *et al.* 1996; Popay 2012). The potato mirid (*Calocoris norvegicus*) and bluegreen lucerne aphid (*Acyrthosiphon kondoi*) have also been shown to cause significant reduction to *Trifolium repens* seed production in the Canterbury region of NZ (Schroeder 1998) and the caterpillars of NZ-endemic porina moths (*Wiseana* spp.) also feed on *Trifolium* (Zydenbos *et al.* 2011; Popay 2012). Slugs are another major pest of clover, and the molluscicidal baiting of pastures in NZ has been shown to increase clover yield by 12–40% (Barker & Addison 1992; Wilson & Barker 2010).

Nevertheless, worldwide*, Trifolium* have been documented to be host to an abundance of pathogens and herbivores (Gibbs, Varma & Woods 1966; Barnett & Diachun 1985; Leath 1985; Manglitz 1985; Cook *et al.* 1992). Despite the presence of many well-known clover enemies in NZ, it is likely that some enemy taxa found in the native range are still absent from NZ. An additional species of clover root weevil, *Sitona lineatus*, that causes significant damage to clover in UK pastures (Clements & Murray 1991), for example, has not yet reached NZ (Gerard 2001). The acquisition of novel enemies in NZ, such as the endemic clover root weevil, mānuka scarab beetle and Porina moth or Australian field cricket, may in fact override the benefits provided by escaping a proportion of native-range enemies.

Lastly, alien plant populations may evolve post-establishment in response to selection pressures in their new environment. The Evolution of Increased Competitive Ability (EICA) hypothesis states that populations of alien species can evolve by losing costly defensive traits in response to an absence of natural enemies (Blossey & Nötzold 1995). This possibility was excluded in the PSF experiment in Chapter 3 by using seed of native-range provenance, but a parallel study is investigating this phenomenon with NZ-naturalised *Trifolium* (Shelby, in prep.).

Many of these potential determinants of invasive success are not mutually exclusive, and successful invasion is likely be the product of a combination of interacting contributory factors. Suding *et al.* (2013), for example, indicated that empirically-measured PSF responses could not solely explain the ability of plant invaders to out-compete native species, but invader dominance was greatly facilitated when additional factors such as disturbance or propagule dispersal were included in their computer simulation.

6.4 Implications

This study provides some valuable insights into how soil biota may affect the plant invasion process. The findings highlight that the strength of escape from inhibitory PSF is species-specific and that PSF responses should not be extrapolated to other closely related plant species in the same geographic region (i.e. if one alien plant species experiences a release from inhibitory PSF in a given region, it does not necessarily mean that fellow alien congeners also benefit). If the accumulation of inhibitory PSF by longer-established and more widespread alien plant populations is a common phenomenon, it may impose some control on the performance of invasive plants over time and provide a potential explanation for the boom and bust dynamics of some alien plant populations. Conversely, the trend for a transient escape from inhibitory PSF did not hold in the present study. Given that there is only one multi-species test supporting the hypothesis of a transient escape from inhibitory PSF (Diez *et al.* 2010), the generalisability of the phenomenon is questioned. From a management perspective, the accumulation of inhibitory PSF could be used to justify a 'do nothing' approach to the control of invasive plants (Strayer 2012; Dostál *et al.* 2013), so determining the generalisability of the phenomenon is important.

The novel statistical approach for calculating PSF ratios outlined in Chapter 2 represents an improvement on current PSF calculation methods as it can handle unbalanced and non-independent data. It may also be useful to statistically account for confounding plant growth covariates, such as aboveground enemy damage, but its real potential lies in its ability to statistically tease apart components of the PSF black box. Future PSF experiments may be conducted to use the approach to

its full potential by quantifying soil-borne mutualist colonisation and using the approach to provide a novel insight into the relative contribution of different soil biota components to plant growth. Suitable data from previous PSF experiments that scored plant growth covariates may also allow retrospective application of the statistical approach.

In terms of agricultural significance, the findings suggest that agricultural *Trifolium* species do not grow better when exposed to NZ soil biota relative to European soil biota. The soil used in the PSF experiments was sampled at sites that were not agricultural pastures, so it is possible that PSF responses inside pastures are even more suppressive to *Trifolium* growth due to factors such as higher *Trifolium* densities. The selection and application of plant growth-promoting soil-borne endophytes (Dudeja *et al.* 2012) may be a way in which PSF can be made more favourable to *Trifolium* growth and productivity in NZ pastures. Such endophytes have been applied to protect pasture ryegrass (*Lolium* spp.) from insect attack in NZ (Popay & Hume 2011). In addition, the finding that more geographically restricted, non-agricultural, *Trifolium* species experience stronger escape from inhibitory PSF in NZ may suggest that the agricultural productivity of clover-based pastures may benefit from diversifying to use other *Trifolium* species. Most of the species in the group that experienced stronger escape from inhibitory PSF are annuals, and many of these species, such as *T. campestre*, were also shown to have access to suitable rhizobia in naturalised sites in NZ. The use of these clover species in situations where *T. repens* and *T. pratense* are less successful, such as in dry, semi-arid grasslands (see Boswell *et al.* 2003), may therefore improve agricultural productivity in these areas. Nevertheless, some of the annual *Trifolium* that appear to benefit from escaping a degree of inhibitory PSF are probably not amenable to productive pastures due to their small size (e.g., *T. micranthum* and *T. ornithopodioides*). Lastly, the finding that clover rhizobia in NZ soils appear to be as symbiotically effective and genetically diverse as strains from the native range suggests that rhizobial inoculation of agricultural *Trifolium* seed may not be required in many cases in NZ, as suggested by Lowther & Kerr (2011).

6.5 Future research and recommendations

6.5.1 Escape from inhibitory PSF and temporal dynamics of PSF

Further tests of whether the escape from inhibitory PSF is transient using a range of alien plant species are needed. In the future, this could allow predictions to be made concerning which species are most likely to accumulate inhibitory PSF that controls their performance. There may, for example, be differences in the likelihood of plant species accumulating inhibitory PSF based on physiological traits (e.g., annual versus perennial species).

The temporal dynamics of PSF processes in general are poorly understood (Kardol *et al.* 2013) and longer-term experiments tracking PSF responses over time would provide valuable insight. A study quantifying the strength of PSF experienced by an alien plant species from initial colonisation of a site through to alien dominance would be particularly informative. This would likely require glasshouse-based experiments under controlled conditions using field-sampled soil, but may be combined with field-based measurements of plant performance over the course of invasion, in order to identify whether temporal variability in the strength of PSF is correlated with the success of the invader in the field. This type of experiment may also identify whether the strength of PSF fluctuates over successive years. The escape from inhibitory PSF may, for instance, be confined to windows in certain years in which alien plants can greatly increase biomass, seedling recruitment or seed set. These long-term studies are, however, likely to be logistically challenging, and further studies of PSF responses over invasion chronosequences, such as the study by Dostál *et al.* (2013), are a valuable and possibly more feasible alternative. In addition, if naturally-occurring chronosequences cannot be located, it could be possible to experimentally create them by establishing a plant species in different plots over consecutive years to form a gradient of time since establishment.

The accumulation of soil-borne mutualists may also contribute to temporal dynamics in PSF. Modelling has indicated that threshold densities of suitable soil-borne mutualists need to be attained to allow plant invasion (Parker 2001), and this may contribute to a 'lag phase' before alien plant populations become invasive. Jin *et al.* (2004) also found that AMF species richness and AMF infection of the plant *Solidago canadensis* increased with longer invasion in China. It is thus possible that PSF at a site becomes increasingly more positive for alien plants during the first few years of colonisation as optimal soil-borne mutualists accumulate, but then as time progresses, become increasingly negative due to the accumulation of soil-borne enemies. The statistical approach developed in Chapter 2 may be applied to elucidate the relative effect of mutualistic and antagonistic soil biota over an invasion chronosequence of a legume species by quantifying rhizobial and mycorrhizal colonisation and PSF responses.

PSF experiments such as those described would benefit from the parallel application of molecular tools to provide information about the specific taxonomic differences in soil communities. Quantitative-PCR (QPCR) may be particularly useful (e.g., Nacke *et al.* 2011), as this technique can be used to estimate the relative abundance of different taxonomic groups of soil organisms (e.g., Rousk *et al.* 2010). This may be applied to identify differences in the composition of soil communities associated with a plant species in the introduced and native range, or identify taxonomic changes in soil communities that cause increasingly inhibitory PSF.

6.5.2 Negative controls

The general observation that most invasive plants have been capable of acquiring suitable mutualists (Richardson *et al.* 2000a) may not be surprising, since these species have successfully established and are thus less likely to have experienced a loss of mutualists. Documenting whether a lack suitable mutualists has presented a barrier to the establishment of alien plants that failed to persist would provide a more representative view of the importance of mutualists in the naturalisation process. The present study may be extended to examine whether *Trifolium* species that were introduced to NZ but failed to establish lack suitable rhizobia strains in NZ soils. Although all *Trifolium* native to the UK have successfully established in NZ, broadening the European sampling region would allow inclusion of species documented as introduced to NZ (and permitted for import of seed) but not naturalised (e.g., *T. affine* and *T. alexandrinum*).

6.5.3 Replication in plant-soil feedback experiments

Plants grown from a single seed source in soil from one location are likely to vary considerably in their biomass. Quantifying PSF at field sites based on one PSF ratio, which usually equates to a ratio between two plants, may therefore be imprecise. It would be sensible for future PSF studies to include replication at the lowest treatment level, for instance, to include a sufficient number of within-field site replicates (i.e., 5 or more), when sites are considered true replicates of PSF responses. Although these within-site replicates are not independent, they can be handled by the statistical approach developed in Chapter 2, and provide more accurate estimates of PSF responses at each site.

6.5.4 Multitrophic interactions and dynamic enemy release

A multitrophic perspective may provide an improved understanding of how enemy release affects plant performance in the field. Enemies that catch-up with alien plants from their native range are likely to experience a dynamic enemy release effect themselves that allows them to be particularly effective at imposing control on alien plant performance initially (Keane & Crawley 2002), but this control may then attenuate as enemies of that enemy gradually catch up over time. For instance, parasitoids associated with alien herbivore hosts have been shown to accumulate with time (Cornell & Hawkins 1993). Alien mutualists may too benefit from the enemy release effect. Fungivorous nematodes that attack mycorrhizal fungi (Bakhtiar *et al.* 2001), for example, may be absent from the introduced range, enhancing the mutualistic benefit of mycorrhizae to alien plant performance. Although experiments investigating this would be challenging, this multitrophic perspective highlights the complex and dynamic nature of the enemy release effect.

6.6 Conclusions

Overall, this study provides mixed evidence for the hypothesis that alien plants escape inhibitory PSF, while all species tested appear to have retained key soil-borne mutualists in their naturalised range. This study provides a well-needed multi-species test of whether the escape from inhibitory soil biota is transient. A hypothesised progressive decline in the strength of escape with longer naturalisation or larger geographic spread of the alien plant species is not supported, and it is therefore unclear whether a transient escape from inhibitory PSF is a common phenomenon. There was, however, evidence that species' agricultural status, geographic spread and the phylogenetic relatedness to other alien species may possibly influence the degree to which alien plants escape inhibitory PSF, although further research is required to tease these factors apart.

This study also shows that less successful alien plant species that are not considered invasive *per se* can experience a significant release from inhibitory PSF, while also retaining rich communities of soil-borne mutualists. Factors other than soil biota are therefore probably more important in determining whether some alien plants become inordinately successful to the extent that they are considered invasive, which emphasises the need to develop multifactor, non-mutually exclusive invasion hypotheses. Lastly, the indications that the strength of escape from inhibitory PSF can be plant species-specific among congeners that occur in the same geographic region highlights the importance of examining multiple alien species when testing plant invasion hypotheses.

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Appendices

Appendix A: R code for Chapter 2

R script 1. R code used to generate the simulated data set and calculate plant-soil feedback via the standard and model approach.

```
# this script uses a small simulated data set to illustrate that
# plant-soil feedback (PSF) ratios can be calculated though a linear
# mixed model framework

# load linear mixed model package
  library(arm)

# set a working directory
  setwd('C:/Users/mcginnk/Desktop/Data')

######################
##  simulate data   ##
######################
# set the random number seed so we get the same result
# every time the script is run
  set.seed(666)

# simulate data according to field site-level variation
# and individual-level random variation
# the site-level effect is the same for the pair of observations
# from each site
  site <- 1:10       # field site numbers
  n.sites <- 10       # total number of field sites
  live.int <- 8       # mean growth of live treatment
  sterile.int <- 5    # mean growth of sterilised treatment
  site_var <- rnorm(n.sites, 0, 1)   # random site-level variation
# growth of plants in live with site & individual variation
  L <- live.int + site_var + rnorm(n.sites, 0, 1)
# growth of plants in sterile with site & individual variation
  S <- sterile.int + site_var + rnorm(n.sites, 0, 1)
```

```
################################
##  standard PSF calculation  ##
################################
# structure of the data for 'pairwise' standard calculation
  pair <- data.frame(site=site, live=L, control=S)

# standard pairwise calculation
# log(live/sterilised, pairwise) where pairwise means matching
# L and S observations from the same field site
  pair$ratio <- log(pair$live/pair$control)

# round to two decimal places
  round(pair, 2)

# mean PSF value over the 10 field sites
  round(mean(pair$ratio), 2)

# 95% confidence intervals (CI) around mean PSF
  se <- sd(pair$ratio)/sqrt(nrow(pair)) # first get standard error
  mean(pair$ratio) + qt(0.975, (nrow(pair)-1)) * se # upper CI
  mean(pair$ratio) - qt(0.975, (nrow(pair)-1)) * se # lower CI

# a paired t-test approach can also replicate
# the standard calculation with efficient use of R script
  t <- t.test(pair$ratio)
  t$estimate # mean PSF
  t$conf.int # confidence intervals

########################################
##  model approach to PSF calculation  ##
########################################
# create data structure
# same data as the standard calculation, arranged differently
  dat <- data.frame(site=c(site, site), tmt=rep(c('Live','Control'),
each=n.sites), growth=c(L,S))
  dat$tmt <- factor(dat$tmt, levels=c('Control', 'Live'))
```

```
  dat$site <- factor(dat$site)

# round growth rates to two decimal places
  dat$growth <- round(dat$growth, 2)

# linear mixed model that calculates PSF accounting for site effect
# (uses a weighted average accounting for site-level variation)
  a <- summary(m1 <- lmer(log(growth) ~ tmt + (1|site), data=dat))
  a

# capture model output
  #capture.output(summary(a), file='model_output.doc', append=TRUE)

# mean PSF value from model
  mean.ratio <- a$coef[2, 1]

# 95% confidence intervals (CI) based on standard errors
# produced by the model
  se2 <- a$coef[2, 2]                          # standard error
  mean.ratio + qt(0.975, (nrow(pair)-1)) * se2 # upper CI
  mean.ratio - qt(0.975, (nrow(pair)-1)) * se2 # lower CI

# compare with the standard approach / t-test approach
  t$conf.int

# alternative simulation-based method to get 95% CIs
# this is useful if doing subsequent manipulations with
# the mean PSF values
# using the vcov matrix:
  mat <- mvrnorm(100000, fixef(m1), vcov(m1))
  quantile(mat[,2], probs=c(0.025, 0.975))
# or using the sim function
# which works slightly differently than the vcov function)
  #mat <- sim(m1, 10000)@fixef
  #quantile(mat[,2], probs=c(0.025, 0.975))

#####################################################
```

```
##  model approach flexible to many PSF equations ##
#################################################
# specifying the model without a soil treatment (tmt) intercept
# produces a mean for each treatment
# having accounted for the site effects
  m2 <- lmer(log(growth) ~ tmt - 1 + (1|site), data=dat)

# simulate 10,000 values that fit into the normal distributions
# of the mean of each soil treatment (live and sterilised)
# and then calculate the alternative PSF equation: log(L)-log(S)
# remember it's already modelled on log scale
# can do this using the vcov function:
  mat <- mvrnorm(100000, fixef(m2), vcov(m2))
# perform the PSF calculation for each row of the 100,000 values
  alt <- mat[, 2] - mat[, 1]
# mean PSF value
  mean(alt)
# 95% confidence intervals
  quantile(alt, probs=c(0.025, 0.975))

  # can alternatively do this using the sim function:
  #mat2 <- sim(m1, 10000)@fixef
  #alt2 <- mat2[, 2] - mat2[, 1]
  #quantile(alt2, probs=c(0.025, 0.975))


# compare this to the standard approach
# for calculating log(L)-log(S)
  pair$alt <- log(pair$live)-log(pair$control)
# mean log(L)-log(S) PSF according to the standard approach
  mean(pair$alt)
  se <- sd(pair$alt)/sqrt(nrow(pair))             # standard error
  mean(pair$alt) + qt(0.975, (nrow(pair)-1)) * se # upper CI
  mean(pair$alt) - qt(0.975, (nrow(pair)-1)) * se # lower CI
```

R script 2. R code used to calculate plant-soil feedback via the model framework and remove the effect of rhizobia for the case study data.

```
#########################
##  Data preparation   ##
#########################
# load linear mixed model package
  library(arm)

# set a working directory
  setwd('C:/Users/mcginnk/Desktop/Data/FB methods')

# read in data
  dat <- read.table('Case study data.txt', sep='\t', quote='',
fill=T, header=T)

# change order of live and sterilised treatments to set live soil as
the baseline category ready for the model
  dat$tmt <- factor(dat$tmt, levels=c('S', 'L'))

# mean nod scores
  tapply(dat$nods2, list(dat$tmt, dat$soil), mean)

#############################################################
##  M3: calculation via model without removing rhizo effect  ##
#############################################################
# separate data sets for UK and NZ soil, as these were conducted
# in different glasshouses and could be considered independent
# studies for calculating feedback ratios
  uk <- subset(dat, soil=='UK')
  uk$tmt <- factor(uk$tmt, levels=c('S', 'L'))
  uk$site <- factor(uk$site)
  table(uk$site)

  nz <- subset(dat, soil=='NZ')
  nz$tmt <- factor(nz$tmt, levels=c('S', 'L'))
  nz$site <- factor(nz$site)
  table(nz$site)
```

```
# fit a model that computes the difference in growth rate
# between treatments on the log scale
# [log(L) - log(S)] is equal to the equation log(L/S)
# do this for NZ and UK soil separately
  summary(m3u <- lmer(log(growth_rate) ~  tmt + (1|site), data=uk,
REML=TRUE))
  summary(m3n <- lmer(log(growth_rate) ~  tmt + (1|site), data=nz,
REML=TRUE))

# estimate CIs by simulation from the fitted model
  mat3u <- mvrnorm(100000, fixef(m3u), vcov(m3u))
  q3u <- quantile(mat3u[,2], probs=c(0.025, 0.25, 0.75, 0.975))
  mat3n <- mvrnorm(100000, fixef(m3n), vcov(m3n))
  q3n <- quantile(mat3n[,2], probs=c(0.025, 0.25, 0.75, 0.975))

# combine into a table
  q3u <- c(q3u, fixef(m3u)[2])
  q3n <- c(q3n, fixef(m3n)[2])
  MFB <- data.frame(rbind(q3n, q3u))
  colnames(MFB) <- c('lcl', 'flcl', 'fucl', 'ucl', 'FB')
  MFB$method <- c('Model 1', 'Model 1')
  MFB$soil <- c('NZ', 'UK')
  MFB

############################################################
##  M4: calculation via model removing the rhizobia effect ##
############################################################
# fit models that include nodulation score as a covariate
  summary(m4u <- lmer(log(growth_rate) ~  tmt + nods2 + (1|site),
data=uk, REML = TRUE))
  summary(m4n <- lmer(log(growth_rate) ~  tmt + nods2 + (1|site),
data=nz, REML = TRUE))

# estimate CIs by simulation from the fitted model
  mat4u <- mvrnorm(100000, fixef(m4u), vcov(m4u))
  q4u <- quantile(mat4u[,2], probs=c(0.025, 0.25, 0.75, 0.975))
```

```
  mat4n <- mvrnorm(100000, fixef(m4n), vcov(m4n))
  q4n <- quantile(mat4n[,2], probs=c(0.025, 0.25, 0.75, 0.975))

# combine into a table
  q4u <- c(q4u, fixef(m4u)[2])
  q4n <- c(q4n, fixef(m4n)[2])
  MFB2 <- data.frame(rbind(q4n, q4u))
  colnames(MFB2) <- c('lcl', 'flcl', 'fucl', 'ucl', 'FB')
  MFB2$method <- c('Model 2', 'Model 2')
  MFB2$soil <- c('NZ', 'UK')
  MFB2

############################################################
##  Propagating uncertainty onto subsequent calculations  ##
############################################################
# illustrate how to perform calculation with PSF values
# from the model and propagate the uncertainties around them
# by calculating the difference between mean PSF in NZ and UK soil
# calculate 100,000 possible values for the biogeographic difference
# using values from model M2
  BG <- mat4n[,2] - mat4u[,2]
# mean biogeographic difference and CIs
  mean(BG)
  quantile(BG, probs=c(0.025, 0.25, 0.75, 0.975))

# use the same approach to calculate the difference between PSF
# values with and without rhizo i.e. the difference between PSF
# values calculated in the M3 and M4 models to quantify the relative
contribution of rhizobia to PSF
# for UK soil
  relUK <- mat3u[,2] - mat4u[,2]
  mean(relUK)
  quantile(relUK, probs=c(0.025, 0.25, 0.75, 0.975))
# for NZ soil
  relNZ <- mat3n[,2] - mat4n[,2]
  mean(relNZ)
  quantile(relNZ, probs=c(0.025, 0.25, 0.75, 0.975))
```

Appendix B: Supporting information for Chapter 2

Table B.1. Structure of the simulated data for the standard pairwise plant-soil feedback calculation. The data were simulated based on a mean of 8 for live soil and a mean of 5 for sterilised soil, both including variation randomly drawn from a normal distribution with mean = 0 and standard deviation = 1.

Field site number	Live soil treatment growth	Control soil treatment growth	Feedback ratio = log(live/control)
1	10.9	5.06	0.77
2	8.24	5.83	0.35
3	8.51	5.91	0.36
4	8.31	6.72	0.21
5	5.92	2.81	0.74
6	8.68	4.28	0.71
7	7.55	2.57	1.08
8	7.54	2.43	1.13
9	5.63	2.15	0.96
10	8.74	3.61	0.88

Table B.2. Structure of the simulated data for the pairwise plant-soil feedback calculation via the model framework.

Field site number	Soil treatment	Growth
1	Live	10.9
2	Live	8.24
3	Live	8.51
4	Live	8.31
5	Live	5.92
6	Live	8.68
7	Live	7.55
8	Live	7.54
9	Live	5.63
10	Live	8.74
1	Control	5.06
2	Control	5.83
3	Control	5.91
4	Control	6.72
5	Control	2.81
6	Control	4.28
7	Control	2.57
8	Control	2.43
9	Control	2.15
10	Control	3.61

Table B.3: Data for calculation of plant-soil feedback for the case study example.

species	seed	soil	rhizo	tmt	rep	site	growth_rate	nods2
G	NZ	UK	1	L	1	u1	0.020154	1
G	NZ	UK	0	L	1	u1	0.016564	1
G	NZ	UK	1	L	2	u4	0.018564	1
G	NZ	UK	0	L	2	u4	0.018205	1
G	NZ	UK	1	L	3	u10	0.032949	2
G	NZ	UK	0	L	3	u10	0.026615	3
G	NZ	UK	1	L	4	u12	0.025692	1
G	NZ	UK	0	L	4	u12	0.021564	2
G	NZ	UK	1	L	5	u17	0.020103	1
G	NZ	UK	0	L	5	u17	0.015974	0
G	NZ	UK	1	S	1	u1	0.024179	3
G	NZ	UK	0	S	1	u1	0.015103	0
G	NZ	UK	1	S	2	u4	0.018103	3
G	NZ	UK	0	S	2	u4	0.011179	0
G	NZ	UK	1	S	3	u10	0.029077	2
G	NZ	UK	0	S	3	u10	0.02141	0
G	NZ	UK	1	S	4	u12	0.018974	1
G	NZ	UK	0	S	4	u12	0.020846	0
G	NZ	UK	1	S	5	u17	0.029436	2
G	NZ	UK	0	S	5	u17	0.021103	0
G	UK	UK	1	L	1	u1	0.029231	1
G	UK	UK	0	L	1	u1	0.023487	1
G	UK	UK	1	L	2	u4	0.028564	3
G	UK	UK	0	L	2	u4	0.022308	2
G	UK	UK	1	L	3	u10	0.036487	2
G	UK	UK	0	L	3	u10	0.034333	2
G	UK	UK	1	L	4	u12	0.030128	3
G	UK	UK	0	L	4	u12	0.038795	2
G	UK	UK	1	L	5	u17	0.032974	2
G	UK	UK	0	L	5	u17	0.026564	3
G	UK	UK	1	S	1	u1	0.033385	3
G	UK	UK	0	S	1	u1	0.021615	0
G	UK	UK	1	S	2	u4	0.024795	3
G	UK	UK	0	S	2	u4	0.025692	2
G	UK	UK	1	S	3	u10	0.033128	3
G	UK	UK	0	S	3	u10	0.044	0
G	UK	UK	1	S	4	u12	0.041564	2
G	UK	UK	0	S	4	u12	0.023333	0
G	UK	UK	1	S	5	u17	0.040179	3
G	UK	UK	0	S	5	u17	0.033051	0
G	NZ	NZ	1	L	1	n5	0.02029	2
G	NZ	NZ	0	L	1	n5	0.031014	2
G	NZ	NZ	1	L	2	n6	0.017246	2

G	NZ	NZ	0	L	2	n6	0.021014	2
G	NZ	NZ	1	L	3	n8	0.01058	1
G	NZ	NZ	0	L	3	n8	0.003043	0
G	NZ	NZ	1	L	4	n9	0.017681	1
G	NZ	NZ	0	L	4	n9	0.011014	1
G	NZ	NZ	1	L	5	n10	0.017681	1
G	NZ	NZ	0	L	5	n10	0.007101	0
G	NZ	NZ	1	S	1	n5	0.016377	2
G	NZ	NZ	0	S	1	n5	0.015507	1
G	NZ	NZ	1	S	2	n6	0.008841	2
G	NZ	NZ	0	S	2	n6	0.01087	1
G	NZ	NZ	1	S	3	n8	0.005652	1
G	NZ	NZ	0	S	3	n8	0.005652	1
G	NZ	NZ	1	S	4	n9	0.01087	1
G	NZ	NZ	0	S	4	n9	0.012899	0
G	NZ	NZ	1	S	5	n10	0.013043	2
G	NZ	NZ	0	S	5	n10	0.012609	1
G	UK	NZ	1	L	1	n5	0.017391	3
G	UK	NZ	0	L	1	n5	0.024928	2
G	UK	NZ	1	L	2	n6	0.021159	2
G	UK	NZ	0	L	2	n6	0.029275	3
G	UK	NZ	1	L	3	n8	0.015362	3
G	UK	NZ	0	L	3	n8	0.011014	1
G	UK	NZ	1	L	4	n9	0.021014	3
G	UK	NZ	0	L	4	n9	0.025942	2
G	UK	NZ	1	L	5	n10	0.015217	2
G	UK	NZ	0	L	5	n10	0.007246	2
G	UK	NZ	1	S	1	n5	0.024058	2
G	UK	NZ	0	S	1	n5	0.008696	1
G	UK	NZ	1	S	2	n6	0.01971	2
G	UK	NZ	0	S	2	n6	0.008116	2
G	UK	NZ	1	S	3	n8	0.016087	2
G	UK	NZ	0	S	3	n8	0.008986	1
G	UK	NZ	1	S	4	n9	0.013333	2
G	UK	NZ	0	S	4	n9	0.022609	2
G	UK	NZ	1	S	5	n10	0.01913	2
G	UK	NZ	0	S	5	n10	0.012029	2

Table B.4. R code using the lmer function of the R package arm (Gelman & Su 2014) to fit linear mixed models and calculate plant-soil feedback. See Table B.7 for explanations of each variable.

Model	R code
Model M1 (simulated data)	lmer(log(growth)) ~ tmt + (1|site) , data=dat)
Model M2 (simulated data)	lmer(log(growth)) ~ tmt-1 + (1|site) , data=dat)
Model M3 (case study)	lmer(log(growth rate) ~ tmt + (1|site) , data=dat)
Model M4 (case study)	lmer(log(growth rate) ~ tmt + nods + (1|site) , data=dat)

Table B.5. Output statistics from linear mixed model M1. The 'tmtLive' estimate gives the mean plant-soil feedback value and the p value for this estimate indicates whether there is a significant difference between live and sterilised soil.

Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
intercept	1.34	0.10	13.05	
tmtLive	0.72	0.10	7.16	< 0.001
Random effects				
	Variance	**Std. Dev.**		
site	0.06	0.24		
residual	0.05	0.23		

Table B.6. Output statistics from linear mixed model M2. Plant-soil feedback here can be calculated from the difference between the 'tmtControl' and 'tmtLive' estimates.

Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
tmtControl	1.34	0.10	13.05	
tmtLive	2.06	0.10	20.05	< 0.001
Random effects				
	Variance	**Std. Dev.**		
Site	0.06	0.24		
residual	0.05	0.23		

Table B.7. Breakdown of the variables included in linear mixed models M1-M4 used to calculate plant-soil feedback (PSF), according to the R code given in Table B.4.

Model variable	Fixed or random effect	Models used	Explanation
Tmt	Fixed	All models	Soil treatment (live biota = 0 or sterilised = 1), **with** a constant intercept term. Models specifying such a binary variable as a fixed effect produce fixed effect coefficients that are expressed as the difference between the mean of the two variable categories (in our examples: live – sterilised soil).
tmt-1	Fixed	M2	Soil treatment (live biota = 0 or sterilised = 1), **without** a constant intercept term. Models specifying this produce fixed effect coefficients that are expressed as separate means of the response variable for the fixed effect treatment categories (in our examples, separate estimates of growth rates for live and sterilised soil that can be used to calculate PSF from many different PSF equations).
Nods	Fixed	M3, M4	Nodulation score: a variable that quantified the degree of nodulation by nitrogen-fixing rhizobia for each plant. Specifying this effect modelled linear regressions between plant growth rate and nodulation score. The fixed effect coefficients of the fitted models then represented estimates of PSF at a nodulation score of zero, thereby statistically removing the effect or rhizobia. Figure 2.2 illustrates the model regressions between growth and nodulation.
(1|site)	Random	M1-M4	Field site for soil collection. Allows for the 'pairwise' nature of the sampling scheme/experimental design by grouping observations of the same field soil origin by allowing the model intercept coefficients to vary by these groups.

Table B.8. Output statistics from linear mixed model M3. The 'tmt' fixed effect estimates (underlined) give the plant-soil feedback value. The p value for the tmt variable indicates whether there is a significant difference in plant growth rates between live and sterilised soil.

UK soil				
Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
Intercept	-3.685	0.089	-41.23	
Tmt	<u>0.001</u>	0.089	0.01	0.995
Random effects				
	Variance	**Std. Dev.**		
Site	0.02	0.14		
residual	0.08	0.28		
NZ soil				
Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
intercept	-4.40	0.15	-28.53	
Tmt	<u>0.22</u>	0.13	1.64	0.11
Random effects				
	Variance	**Std. Dev.**		
site	0.07	0.27		
residual	0.18	0.42		

Table B.9. Output statistics from linear mixed model M4 used to statistically remove the effect of rhizobia from the plant-soil feedback (PSF) responses. The 'tmt' fixed effect estimates (underlined) give the PSF value. The p value for the tmt variable indicates whether there is a significant difference between the plant growth rate in live and sterilised soil, and the p value for the 'nods' variable indicates whether there is a significant relationship between nodulation score and plant growth rate.

UK soil				
Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
intercept	-3.86	0.1	-38.59	
Tmt	-0.05	0.08	-0.59	0.56
Nods	0.13	0.03	3.74	< 0.001
Random effects				
	Variance	**Std. Dev.**		
Site	0.02	0.16		
residual	0.06	0.24		
NZ soil				
Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
intercept	-4.91	0.17	-29.24	
Tmt	0.14	0.11	1.20	0.24
Nods	0.34	0.08	4.33	< 0.001
Random effects				
	Variance	**Std. Dev.**		
Site	0.04	0.20		
residual	0.12	0.35		

Appendix C: Full methodology for case study data (Chapter 2)

The case study data originated from an experiment designed to quantify the effect of soil biota on the growth of *Trifolium glomeratum* in the introduced range of New Zealand and native range of the United Kingdom. Due to logistical limitations to transferring soil, separate glasshouse experiments were conducted within each range (at Lincoln University, NZ with NZ soil, and at the Netherlands Institute of Ecology (NIOO-KNAW) with UK soil). Plant-soil feedback was quantified with each range by comparing plant growth in treatments with soil biota (live) relative to controls without (sterilised).

Soil was collected from five separate populations of *Trifolium glomeratum* in both NZ (Banks Peninsula and Christchurch) and in the UK (south coast of England). At each population, soil was collected from the rhizosphere of 10 plants at least 1 m apart. Soil from each population was sieved to 4 mm, bulked and homogenised to form five independent replicates of soil communities from each range. One half of the soil from each population was sterilised. In Europe, soil sterilisation was achieved by gamma irradiation (> 25 KGray, Ede Isotron, The Netherlands), and in NZ, by twice autoclaving on a cycle held at 121^{o}C for 20 min with a minimum of 48 h between runs. To dilute abiotic differences among the field-collected soils, the live or sterilised soil inoculants comprised only 10% of the soil volume in each 1 L plant pot in the glasshouse. The remaining 90% volume of each pot was comprised of a homogeneous and sterilised 'background' soil.

A rhizobia treatment was included consisting of supplement or no supplement of commercial rhizobia (HiStick® for *Trifolium*, Becker Underwood), in addition to a seed treatment consisting of seed of UK or NZ provenance. There were 80 observations in total: 2 soil provenances (NZ, UK) x 5 field soil collection sites x 2 soil biota treatments (live or sterilised) x 2 seed provenances (NZ, UK) x 2 rhizobia treatments (with or without commercial rhizobia).

Seeds were surface-sterilised for 2 min in a solution of 10% household sodium hypochlorite with a drop of Tween 20 surfactant, germinated in sterilised glass beads (Europe) or sand (NZ), and similar-sized seedlings were transplanted into pots inoculated with field soil in the glasshouse. Plants grew at different rates in each glasshouse, so the development of flower buds was used as a harvest cue in each glasshouse. Roots and shoots were separated, oven dried at 70^{o}C for at least 48 h and then weighed.

To evaluate the degree of effective nitrogen-fixation by rhizobia, root nodulation of each plant was scored at the time of harvest on a 0-3 scale adapted from Thrall et al. (2007) and Corbin et al. (1977), based on the size, number, position and colour of nodules. Functional nitrogen-fixing nodules are pink due to the presence of the pigmented-protein leghaemoglobin, whereas ineffective nodules are white (Somasegaran & Hoben 1994). Nodule position on the root system indicates the plant growth-stage at which rhizobia infection occurred. A score of zero represented no nodulation or white nodules only. Scores of 1-3 represented increasing degrees of effective N-fixation: 1 for a low number of small (< 1mm) pink nodules predominantly on lower roots; 2 for an intermediate number of larger pink nodules (many >1mm), some of which at the root crown (top 2 cm of the root system); and 3 for abundant large (many > 1mm) pink nodules, particularly at the root crown.

Appendix D: Field soil sampling sites (Chapter 3)

Table D.1. Geographic locations of populations of each *Trifolium* species (A = *T. arvense*, C = *T. campestre*, D = *T. dubium*, F = *T. fragiferum*, G = *T. glomeratum*, M = *T. micranthum*, P = *T. pratense*, R = *T. repens*, S = *T. striatum*, O = *T. ornithopodioides*, T = *T. tomentosum*) from which soil was collected in New Zealand (NZ), United Kingdom (UK) and Spain (SP). The experiment year column states the year that the field site was used in glasshouse experiments (1=2012; 2=2013). Permission for soil collection from sites with conservation designation in the UK was granted by the relevant authority (sites u15, u17, u28, u18 were 'sites of special scientific interest').

Species	Country	Site reference	Replicate	Site location	Latitude	Longitude	Experiment year
A	NZ	n1	1	Christchurch: railway side near Pope Street	-43.536548	172.609818	NZ1; NZ2
A	NZ	n2	2	Little River, Banks Peninsula	-43.769456	172.790641	NZ1
A	NZ	n6	2	Kaitorete Spit, View Hill Road, Banks Peninsula	-43.825509	172.698964	NZ2
A	NZ	n6	3	Kaitorete Spit, View Hill Road, Banks Peninsula	-43.825509	172.698964	NZ1
A	NZ	n7	3	Poranui Beach Road, Birdlings Flat	-43.815543	172.699996	NZ2
A	NZ	n7	4	Poranui Beach Road, Birdlings Flat	-43.815543	172.699996	NZ1
A	NZ	n9	4	Roadside at Bridge Street, New Brighton	-43.525545	172.722663	NZ2
A	NZ	n9	5	Roadside at Bridge Street, New Brighton	-43.525545	172.722663	NZ1
A	NZ	n30	5	ANZAC-South Brighton Road	-43.519559	172.719673	NZ2
C	NZ	n1	1	Christchurch: railway side near Pope Street	-43.536548	172.609818	NZ2
C	NZ	n4	1	Chorlton Road, Banks Peninsula	-43.675339	173.044438	NZ1
C	NZ	n4	2	Chorlton Road, Banks Peninsula	-43.675339	173.044438	NZ2
C	NZ	n8	2	Western Valley Road, Banks Peninsula	-43.746963	172.795562	NZ1
C	NZ	n8	3	Western Valley Road, Banks Peninsula	-43.746963	172.795562	NZ2
C	NZ	n14	3	Streeters Road	-43.736169	172.625697	NZ1
C	NZ	n14	4	Streeters Road	-43.736169	172.625697	NZ2
C	NZ	n26	4	Shadbolts Road	-43.726109	172.876636	NZ1
C	NZ	n25	5	Big Hill Road, Banks Peninsula	-43.701702	173.064850	NZ1; NZ2
D	NZ	n5	1	Chorlton Road, Okains Bay, Banks Peninsula	-43.704496	173.047237	NZ2
D	NZ	n3	1	Bossu Road, Banks Peninsula	-43.802361	172.841277	NZ1
D	NZ	n8	2	Western Valley Road, Banks Peninsula	-43.746963	172.795562	NZ1; NZ2
D	NZ	n12	3	Lake Forsyth marsh	-43.809746	172.723392	NZ1; NZ2

D	NZ	n23	4	Bridleway, Governors Bay	-43.632091	172.652055	NZ1; NZ2
D	NZ	n24	5	Ataahua, Highway 75	-43.776084	172.645663	NZ1
D	NZ	n38	5	Gravel track near Squally Bay, Banks Peninsula	-43.867156	172.900507	NZ2
F	NZ	n11	1	Recreation field, Park Road, Motukarara	-43.729049	172.584264	NZ2
F	NZ	n12	2	Lake Forsyth marsh	-43.809746	172.723392	NZ2
F	NZ	n16	3	Roadside in Teddington, Gebbies Pass Road	-43.672115	172.658906	NZ2
F	NZ	n35	4	Rountree off Waimairi Road	-43.527334	172.575047	NZ2
F	NZ	n41	5	Streeters Road, Banks Peninsula	-43.734425	172.625366	NZ2
G	NZ	n5	1	Chorlton Road, Okains Bay, Banks Peninsula	-43.704496	173.047237	NZ1; NZ2
G	NZ	n6	2	Kaitorete Spit, View Hill Road, Banks Peninsula	-43.825509	172.698964	NZ1; NZ2
G	NZ	n8	3	Western Valley Road, Banks Peninsula	-43.746963	172.795562	NZ1; NZ2
G	NZ	n9	4	Roadside at Bridge Street, New Brighton	-43.525545	172.722663	NZ1; NZ2
G	NZ	n10	5	Hammerton Road, near Sumner	-43.571216	172.711755	NZ1
G	NZ	n22	5	Heathcote Quarry Track at Major Hornbrook Road, Mount Pleasant	-43.571865	172.716757	NZ2
M	NZ	n31	1	Knowles Street, Mariehau, Christchurch	-43.498280	172.638734	NZ2
M	NZ	n33	2	South Brighton Park near shed	-43.532373	172.734479	NZ2
M	NZ	n34	3	Lincoln University campus	-43.643507	172.468487	NZ2
M	NZ	n35	4	Rountree off Waimairi Road	-43.527334	172.575047	NZ2
M	NZ	n36	5	Kildare Terrace, Lincoln	-43.640650	172.485515	NZ2
O	NZ	n2	1	Little River, Banks Peninsula	-43.769456	172.790641	NZ2
O	NZ	n12	2	Lake Forsyth marsh	-43.809746	172.723392	NZ2
O	NZ	n27	3	Marsh at Governors Bay Teddington Road, Teddington	-43.651829	172.656384	NZ2
O	NZ	n28	4	Cliff edge above Squally Bay, Banks Peninsula	-43.893924	172.914841	NZ2
O	NZ	n34	5	Lincoln University campus	-43.643507	172.468487	NZ2
P	NZ	n13	1	Highway 75 roadside	-43.774485	172.649909	NZ1; NZ2
P	NZ	n15	2	Roadside, Gebbies Pass Road	-43.692179	172.637368	NZ1; NZ2
P	NZ	n17	3	Roadside, Governors Bay Teddington Road, Teddington	-43.651543	172.655799	NZ1; NZ2
P	NZ	n19	4	Bossu Road, Banks Peninsula	-43.802361	172.841277	NZ1; NZ2
P	NZ	n21	5	Days Road, Springston	-43.645604	172.437436	NZ1; NZ2
R	NZ	n1	1	Christchurch: railway side near Pope Street	-43.536548	172.609818	NZ1
R	NZ	n2	1	Little River, Banks Peninsula	-43.769456	172.790641	NZ2
R	NZ	n2	2	Little River, Banks Peninsula	-43.769456	172.790641	NZ1
R	NZ	n4	2	Chorlton Road, Banks Peninsula	-43.675339	173.044438	NZ2

R	NZ	n3	3	Bossu Road, Banks Peninsula	-43.802361	172.841277	NZ1
R	NZ	n5	3	Chorlton Road, Okains Bay, Banks Peninsula	-43.704496	173.047237	NZ2
R	NZ	n4	4	Chorlton Road, Banks Peninsula	-43.675339	173.044438	NZ1
R	NZ	n30	4	ANZAC-South Brighton Road	-43.519559	172.719673	NZ2
R	NZ	n5	5	Chorlton Road, Okains Bay, Banks Peninsula	-43.704496	173.047237	NZ1
R	NZ	n37	5	Field above Squally Bay, Banks Peninsula	-43.887316	172.923772	NZ2
S	NZ	n7	1	Poranui Beach Road, Birdlings Flat	-43.815543	172.699996	NZ2
S	NZ	n22	2	Heathcote Quarry Track at Major Hornbrook Road, Mount Pleasant	-43.571865	172.716757	NZ2
S	NZ	n23	3	Bridleway, Governors Bay	-43.632091	172.652055	NZ2
S	NZ	n24	4	Ataahua, Highway 75	-43.776084	172.645663	NZ2
S	NZ	n25	5	Big Hill Road, Banks Peninsula	-43.701702	173.064850	NZ2
T	NZ	n6	1	Kaitorete Spit, View Hill Road, Banks Peninsula	-43.825509	172.698964	NZ2
T	NZ	n24	2	Ataahua, Highway 75	-43.776084	172.645663	NZ2
T	NZ	n29	3	New Brighton Park lawn	-43.511841	172.716806	NZ2
T	NZ	n30	4	ANZAC-South Brighton Road	-43.519559	172.719673	NZ2
T	NZ	n32	5	Marine Parade, Jervais Rd, South Brighton	-43.516761	172.733834	NZ2
A	SP	s33	1	Parador de Oriel, Aragon, Spain	42.527670	-0.531610	EU1, EU2
A	SP	s36	2	Off A-1606, N of Benabarre, Huerrios Mtns, Aragon, Spain	42.138717	0.476650	EU1, EU2
A	SP	s37	3	Off A-1606, N of Benabarre, Huerrios Mtns II, Aragon, Spain	42.168306	0.451631	EU1, EU2
A	SP	s38	4	Blanes, Catalonia	41.668570	2.766990	EU1
A	SP	s39	5	El Port de la Selva, Catalonia	42.329640	3.199380	EU1, EU2
C	SP	s2	1	A-3312 in Zubillaga, Alaba, Basque Country	42.714920	-2.978400	EU1, EU2
C	SP	s3	2	Off N-1 in Pancorbo, Castilla y León, Spain	42.639080	-3.105910	EU1, EU2
C	SP	s5	3	Barcina del Barco of BU-530 in Castilla y León, Spain	42.781520	-3.228990	EU1, EU2
C	SP	s6	4	Near Rio Ebro, Castilla y León, Spain	42.764480	-3.188760	EU1, EU2
C	SP	s7	5	Embalse de Sobrón, Alaba, Basque Country	42.768070	-3.100810	EU1, EU2
D	SP	s1	1	Estuary in San Kristobal, Biscay, Basque Country	43.368630	2.686820	EU1
D	SP	s21	2	Near crest of Sollube Mtn, Biscay, Basque Country	43.370851	2.763854	EU1
D	SP	s29	3	BI-3332 in Biscay, Basque Country	43.213800	-2.667090	EU1
D	SP	s30	4	Forua, Biscay, Basque Country	43.334030	-2.674990	EU1
D	SP	s31	5	Goitaa/Eibar, Biscay, Basque Country	43.203620	-2.530500	EU1
F	SP	s4	1	Pedrosa de Tobalina, Castilla y León, Spain	42.849300	-3.332480	EU1
F	SP	s5	2	Off BU-532 in Bracina del Barco, Castilla y León, Spain	42.781520	-3.228990	EU1

F	SP	s10	3	Tartales de los Montes, Castilla y León, Spain	42.823300	-3.485990	EU1
F	SP	s12	4	BU-P-5028 in Terminón, Castilla y León, Spain	42.713830	-3.451780	EU1
F	SP	s13	5	Near castle in Poza de la Sal, Castilla y León, Spain	42.669430	-3.507480	EU1
G	SP	s9	1	N-232 in Merindad de Valdivielso, Castilla y León, Spain	42.822210	-3.534000	EU1
G	SP	s11	2	Dirt road in Panizares, Castilla y León, Spain	42.800660	-3.471240	EU1
G	SP	s14	3	Curbo de Burebas, Castilla y León, Spain	42.640800	-3.206540	EU1
G	SP	s17	4	Navas de Bureba, Castilla y León, Spain	42.683040	-3.325160	EU1
G	SP	s18	5	Meadow in Pino de Bureba, Castilla y León, Spain	42.706620	-3.427620	EU1
P	SP	s21	1	Near crest of Sollube Mtn, Biscay, Basque Country	43.370851	2.763854	EU1
P	SP	s24	2	Gorliz, Biscay, Basque Country	43.414760	-2.940180	EU1
P	SP	s25	3	San Juan de Gaztelugatxe, Biscay, Basque Country	43.443310	-2.781430	EU1
P	SP	s26	4	Faro Matxitxako Lighthouse, Biscay, Basque Country	43.455240	-2.752820	EU1
P	SP	s28	5	Junction near camp at Monte Ulia, Gipuzkoa, Basque Country	43.327930	-1.951810	EU1
R	SP	s22	1	Sollube Mtn., Biscay, Basque Country	43.398600	-2.756260	EU1
R	SP	s21	2	Near crest of Sollube Mtn, Biscay, Basque Country	43.370851	2.763854	EU1
R	SP	s23	3	Fika, Biscay, Basque Country	43.320160	-2.816560	EU1
R	SP	s25	4	San Juan de Gaztelugatxe, Biscay, Basque Country	43.443310	-2.781430	EU1
R	SP	s27	5	Near school in Bermeo, Biscay, Basque Country	43.424600	-2.724950	EU1
S	SP	s33	1	Parador de Oriel, Aragon, Spain	42.527670	-0.531610	EU1, EU2
S	SP	s36	2	Off A-1606, N of Benabarre, Huerrios Mtns, Aragon, Spain	42.138717	0.476650	EU1, EU2
S	SP	s40	3	Near Sant Pere de Rodes, Catalonia	42.329330	3.158260	EU1, EU2
S	SP	s41	4	GI-610, Girona, Catalonia	42.291920	3.151930	EU1, EU2
S	SP	s42	5	GI-603 N, Girona, Catalonia	42.364540	3.029260	EU1, EU2
T	SP	s3	1	Off N-1 in Pancorbo, Castilla y León, Spain	42.639080	-3.105910	EU1
T	SP	s8	2	Larrazubi, Alaba, Basque Country	42.748470	-3.050930	EU1, EU2
T	SP	s15	3	Miraveche, Castilla y León, Spain	42.676630	-3.196650	EU1
T	SP	s16	4	Church in Busto de Bureba, Castilla y León, Spain	42.659520	-3.265880	EU1
T	SP	s17	5	Navas de Bureba, Castilla y León, Spain	42.683040	-3.325160	EU1
A	UK	u1	1	Bournemouth, 'Monkey Island'	50.719495	-1.857508	EU1, EU2
A	UK	u6	2	Gosport - Browndown	50.792363	-1.193203	EU1, EU2
A	UK	u11	3	Devon - Off Ilsham Marine Drive, near Torquay	50.458359	-3.491381	EU1, EU2
A	UK	u20	4	Gower - Pennard Burrows	51.576022	-4.091374	EU1
A	UK	u30	4	Swansea marina	51.614266	-3.930945	EU2

A	UK	u22	5	Kenfig	51.515509	-3.727773	EU1, EU2
C	UK	u6	1	Gosport - Browndown	50.792313	-1.193332	EU1
C	UK	u31	1	Gosport - Browndown	50.793469	-1.194668	EU2
C	UK	u28	2	Swansea - Crymlyn	51.624223	-3.843038	EU1
C	UK	u32	2	Gower - near Whiteford Burrows	51.619075	-4.255334	EU2
C	UK	u13	3	Devon - Near Chudleigh	50.614183	-3.623614	EU1, EU2
C	UK	u15	4	Devon - Berryhead	50.395951	-3.491999	EU1
C	UK	u17	4	Devon - headland south of Berryhead	50.382074	-3.499986	EU2
C	UK	u24	5	Kenfig - Near Sharkham Point	51.506086	-3.743090	EU1, EU2
D	UK	u8	1	West Knighton	50.686890	-2.390173	EU1, EU2
D	UK	u10	2	Devon - Off Ilsham Marine Drive, near Torquay	50.459143	-3.489635	EU1
D	UK	u2	2	Bournemouth	50.720274	-1.849621	EU2
D	UK	u13	3	Devon - Near Chudleigh	50.614117	-3.623618	EU1, EU2
D	UK	u19	4	Gower - Port Eynon	51.544036	-4.211099	EU1, EU2
D	UK	u25	5	Gower - Crofty	51.639117	-4.136976	EU1, EU2
F	UK	u3	1	Stanpit Marsh	50.730127	-1.762038	EU1
F	UK	u7	2	Gosport marsh	50.778414	-1.145972	EU1
F	UK	u14	3	Devon - Seaton marsh	50.711478	-3.063522	EU1
F	UK	u25	4	Gower - Crofty marsh	51.639580	-4.137559	EU1
F	UK	u29	5	Llanelli - Pwll	51.684829	-4.202671	EU1
G	UK	u1	1	Bournemouth, ‘Monkey Island’	50.719357	-1.857409	EU1
G	UK	u4	2	Gosport	50.792244	-1.177529	EU1
G	UK	u10	3	Devon - Off Ilsham Marine Drive, near Torquay	50.460213	-3.488661	EU1
G	UK	u12	4	Devon - bank off of Newton Road, near caravan site	50.547119	-3.570686	EU1
G	UK	u17	5	Devon - headland south of Berryhead	50.382074	-3.499986	EU1
M	UK	u2	1	Bournemouth	50.720274	-1.849621	EU1
M	UK	u7	2	Gosport marsh	50.779559	-1.146071	EU1
M	UK	u18	3	Gower - Broadpool	51.597984	-4.151961	EU1
M	UK	u19	4	Gower - Port Eynon	51.543808	-4.211110	EU1
M	UK	u21	5	Gower - Loughour Estuary	51.631163	-4.135005	EU1
O	UK	u2	1	Bournemouth	50.720299	-1.849496	EU1, EU2
O	UK	u5	2	Gosport	50.792120	-1.180596	EU1, EU2
O	UK	u19	3	Gower - Port Eynon	51.543854	-4.211267	EU1, EU2

O	UK	u2	4	Gower - Loughour Estuary	51.630818	-4.134735	EU1, EU2
O	UK	u23	5	Kenfig - old road near Sharkham point	51.506815	-3.740627	EU1, EU2
P	UK	u9	1	Hertfordshire - Ryton	51.981244	-2.388121	EU1
P	UK	u32	1	West Knighton roadside bank	50.687200	-2.381096	EU2
P	UK	u16	2	Devon - Berryhead	50.394077	-3.492447	EU1, EU2
P	UK	u20	3	Gower - Pennard Burrows	51.577112	-4.090455	EU1, EU2
P	UK	u22	4	Kenfig	51.515440	-3.727951	EU1, EU2
P	UK	u25	5	Gower - Crofty	51.638975	-4.136470	EU1, EU2
R	UK	u8	1	West Knighton	50.686878	-2.390506	EU1, EU2
R	UK	u9	2	Hertfordshire - Ryton	51.981209	-2.388349	EU1
R	UK	u2	2	Bournemouth	50.720274	-1.849621	EU2
R	UK	u13	3	Devon - Near Chudleigh	50.614043	-3.623618	EU1, EU2
R	UK	u19	4	Gower - Port Eynon	51.543870	-4.211144	EU1, EU2
R	UK	u26	5	Swansea - Gorseinon park	51.673771	-4.037835	EU1, EU2
S	UK	u1	1	Bournemouth, ‘Monkey Island’	50.719368	-1.858172	EU1, EU2
S	UK	u5	2	Gosport	50.792200	-1.180688	EU1
S	UK	u31	2	Gosport - Browndown	50.793469	-1.194668	EU2
S	UK	u17	3	Devon - headland south of Berryhead	50.382116	-3.499871	EU1, EU2
S	UK	u18	4	Gower - Broadpool	51.597861	-4.151891	EU1, EU2
S	UK	u20	5	Gower - Pennard Burrows	51.575920	-4.091192	EU1, EU2

Appendix E: Properties of background soil (Chapter 3)

Table E.1. Chemical properties of the 'background soil' used in the plant-soil feedback experiments during the two experiment years in New Zealand, before and after sterilisation by double autoclaving at 121° C for 20 min. The background soil formed 90% of the soil volume in each plant pot, into which a 10% inoculation of field soil was made. Autoclaving soil is known to result in larger changes in soil chemistry than sterilisation by gamma irradiation (McNamara *et al.* 2003), potentially causing the release of toxic levels of manganese, in particular (Smith & Smith 1981). These readings, however, verify that no toxic changes occurred. It is still, however, possible that autoclaving altered soil structure and texture. * me = a measure of cation-exchange capacity (milliequivalent of hydrogen per 100 g of dry soil).

	2012 pre-autoclave	2012 post-autoclave	2013 pre-autoclave	2013 post-autoclave
pH	6.3	6.5	5.9	6.1
Olsen P (mg/L)	11	9	10	8
K (me*/100g)	0.33	0.43	0.28	0.45
Ca (me*/100g)	3.9	4.1	2.6	3.4
Mg (me*/100g)	1.08	1.05	0.56	0.66
Na (me*/100g)	0.39	0.46	0.12	0.23
CEC (me*/100g)	7	7	5	6
Total base saturation (%)	82	87	71	76
Mn (mg/kg)	90	83	152	189

Table E.2. Nutrient properties of 'background soil' (see caption above) (NZ = New Zealand glasshouse, EU = Europe glasshouse).

	NZ 2012	NZ 2013	EU 2012
pH	6.50	6.10	6.23
Total N (%)	0.14	0.10	0.06
Total C (%)	1.30	1.20	1.00
C/N ratio	9.40	11.50	16.60
% organic matter	2.30	2.00	2.97

Appendix F: Breakdown of replicates (Chapter 3)

Table F.1. Number of plants from which plant-soil feedback responses were quantified for each *Trifolium* species (A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* F = *T. fragiferum,* G = *T. glomeratum,* M = *T. micranthum,* P = *T. pratense,* R = *T. repens,* S = *T. striatum,* O = *T. ornithopodioides,* T = *T. tomentosum*) for each country of seed origin (UK or Spain) and soil origin (UK, NZ or Spain). The number of observations for each category varied as a consequence of some plants dying and because some species-seed-soil categories were grown in both experiment years (2012 and 2013).

Species / seed origin / soil origin	Number of plants	Number of dead plants	One or two experiment years
A SP NZ	16	4	1
A SP SP	40	0	2
A UK NZ	34	6	2
A UK UK	30	0	2
C SP NZ	17	3	1
C SP SP	36	4	2
C UK NZ	39	1	2
C UK UK	40	0	2
D SP NZ	20	0	1
D SP SP	20	0	1
F SP NZ	19	1	1
F SP SP	20	0	1
F UK NZ	12	6	1
F UK UK	20	0	1
G SP NZ	19	1	1
G SP SP	20	0	1
G UK NZ	40	0	2
G UK UK	20	0	2
M UK NZ	10	0	1
M UK UK	20	0	1
O UK NZ	15	5	1
O UK UK	20	0	1
P SP NZ	20	0	1
P SP SP	20	0	1
P UK NZ	40	0	2
P UK UK	20	0	1
R SP NZ	18	2	1
R SP SP	20	0	1
R UK NZ	20	0	1
R UK UK	20	0	1
S SP NZ	10	0	1
S SP SP	40	0	1
S UK NZ	10	0	1
S UK UK	38	2	2
T SP NZ	10	0	1
T SP SP	20	0	1

Appendix G: Seed sources (Chapter 3)

Table G.1. Sources of seed for each *Trifolium* species (A = *T. arvense*, C = *T. campestre*, D = *T. dubium*, F = *T. fragiferum*, G = *T. glomeratum*, M = *T. micranthum*, P = *T. pratense*, R = *T. repens*, S = *T. striatum*, O = *T. ornithopodioides*, T = *T. tomentosum*), in each soil origin and experiment year (NZ1 and EU1 = 2012; NZ2 and EU2 = 2013). Seed sources: Herbiseed, Twyford, England (H); hand collected in field (HC); Millennium Seed Bank, Royal Botanic Gardens Kew (K); Margot Forde Germplasm Centre, AgResearch, Palmerston North, New Zealand (M); Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales (A).

Species	Soil origin	Experiment year	Seed source / accession number	Seed collection location	Latitude	Longitude
A	UK, NZ	NZ1,EU1,NZ2,EU2	H	Unknown wild British origin	NA	NA
A	SP, NZ	EU1,NZ2,EU2	HC	Spain: Blanes	41.668570	2.766990
C	UK, NZ	NZ1,EU1	K (9405, 9313)	England: Suffolk; Essex	NA	NA
C	UK, NZ	NZ2,EU2	HC	Wales: Crymlyn Burrows	51.624223	-3.843038
C	SP, NZ	EU1,NZ2,EU2	HC	Spain: Gorliz Beach	43.414760	-2.940180
D	SP, NZ	EU1,NZ2	HC	Spain: Embalse de Sobrón, Alaba, Basque Country	43.541990	-5.881340
F	UK, NZ	EU1,NZ2	A (2511)	England: West Sedge Moor, nr Stoke St. Gregory	51.016700	-2.900000
F	SP, NZ	EU1,NZ2	A (882)	Spain: Cindad Real	NA	NA
G	UK, NZ	NZ1,EU1	K (9368)	England: Suffolk	NA	NA
G	UK, NZ	NZ2	HC	England: Bournemouth	50.719357	-1.857409
G	SP, NZ	EU1,NZ2	HC	Spain: El Port de la Selva	42.329640	3.199380
M	UK, NZ	EU1,NZ2	HC	Wales: Port Eynon, Gower	51.543808	-4.211110
O	UK, NZ	EU1,NZ2	HC	Wales: Port Eynon, Gower	51.543808	-4.211110
P	SP, NZ	EU1,NZ2	A (4516)	Spain: Iguanzo, 35 km E of Cangas de Onis	43.304800	-4.835200
P	UK, NZ	EU1,NZ2,NZ3	H	Unknown wild British origin	NA	NA
R	SP, NZ	EU1,NZ2	HC	Spain: Fika	43.320160	-2.816560
R	UK, NZ	NZ1,EU1	K (9335, 86376, 58472, 49579)	England: Essex; Greater London; Wiltshire; East Sussex	NA	NA
S	UK, NZ	EU1,NZ2,EU2	HC	England: Bournemouth	50.719357	-1.857409
S	SP, NZ	EU1,NZ2,EU2	HC	Spain: Huerrios Mtns	42.146660	-0.471590
T	SP, NZ	EU1,NZ2	HC	Spain: Antimio de Arriba	42.561784	-5.773440

Appendix H: Plant growth x nodulation relationships (Chapter 3)

Effective nitrogen-fixing nodules (nodulation scores of 1-3) were formed by 84% and 98% of plants inoculated with live field soil without rhizobia supplement in New Zealand and Europe respectively (Figure H.1). Despite the lower percentage of effective nodulation in NZ soils, all 11 *Trifolium* species demonstrated the ability to form effective nitrogen-fixing nodules without rhizobia supplement in at least some NZ soil. Supplying the commercially-selected rhizobia strain to live field soil caused a small increase in mean nodulation score of 0.01 in Europe and a larger increase of 0.7 in NZ (according to a subset of treatments: from 2012 experiments when the commercial rhizobia treatment was included). This suggests that rhizobia present in field soil were generally capable of forming the same degree of effective nodulation as the commercially-selected strain in Europe, but that the effectiveness of rhizobia available in NZ soils may not be optimal. Unfortunately, due to air or water-borne contamination, effective nitrogen-fixing nodules (scores 1-3) were also present in sterilised soil treatments without rhizobia supplement: 37% in NZ and 49% in Europe (Figure H.1). Since nodulation score was a significant positive linear predictor of plant growth rate ($P < 0.001$, R^2 0.22, DF 897), the presence of rhizobia in sterilised treatments would have confounded net plant-soil feedback calculations. The plant growth benefits provided by nodulation also varied over the two experiment years within each glasshouse (Figure H.2) and among the *Trifolium* species in each glasshouse (Figure H.3).

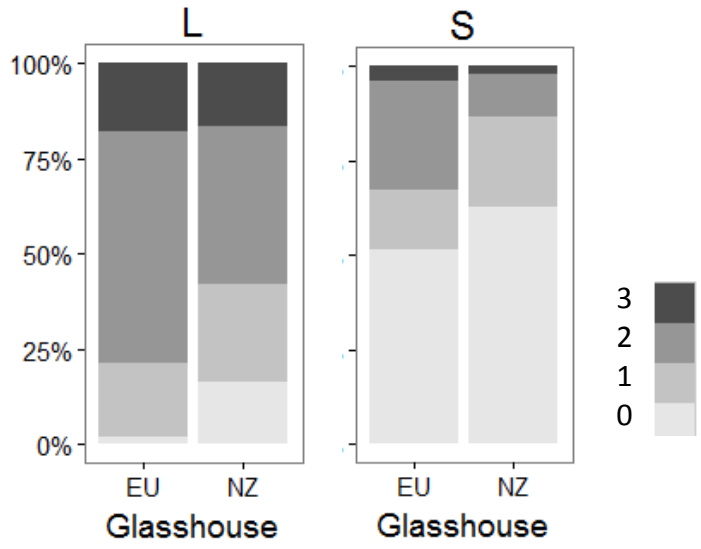

Figure H.1. Nodulation scores that indicated the degree of root nodulation by nitrogen-fixing rhizobia bacteria in live (L) and sterilised (S) field soil, by glasshouse location (EU = Europe; NZ = New Zealand). Scores > 0 indicate effective nitrogen-fixation (see methods section 3.3.4 for full scoring criteria). Only treatments without the addition of commercial rhizobia are included here, in order to illustrate the level of rhizobia contamination in sterilised treatments in both glasshouses.

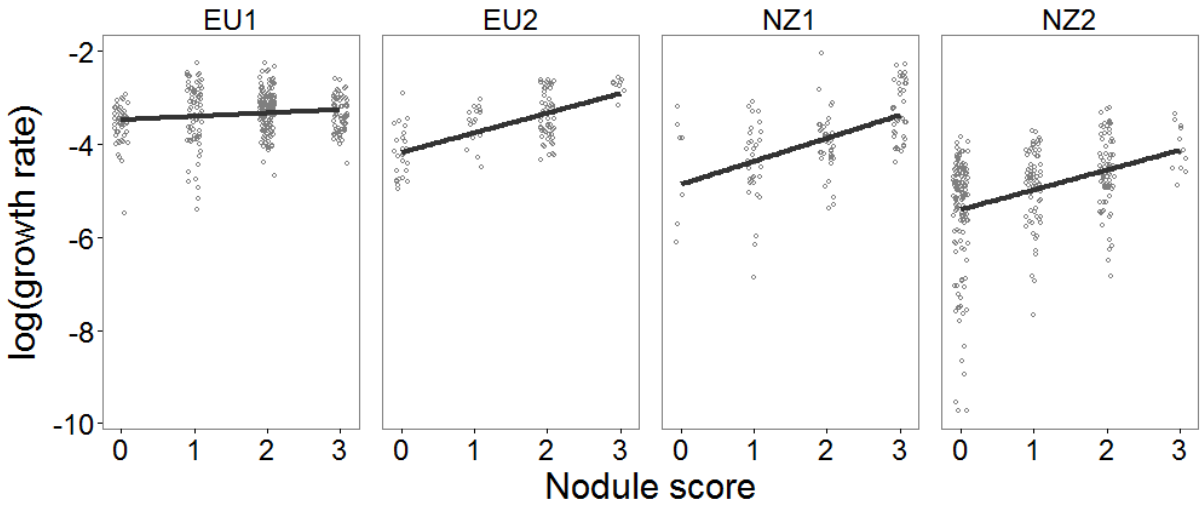

Figure H.2. Linear regressions between nodulation score and plant growth rate, separately for the two experiments within each glasshouse (EU1 = Europe 2012, EU2 = Europe 2013, NZ1 = New Zealand 2012, NZ2 = New Zealand 2013). The respective R^2 values of linear regressions are: 0.02, 0.42, 0.29, 0.14. Slopes were weaker in Europe apparently due to higher intercept values (i.e. growth rate at a nodulation score of 0, with no effective nitrogen-fixation), possibly due to more favourable glasshouse conditions. Given that organic matter and total nitrogen content of background soils were comparable in both glasshouses (Appendix E), it is unlikely that this caused the between-glasshouse differences in the slopes of the relationships.

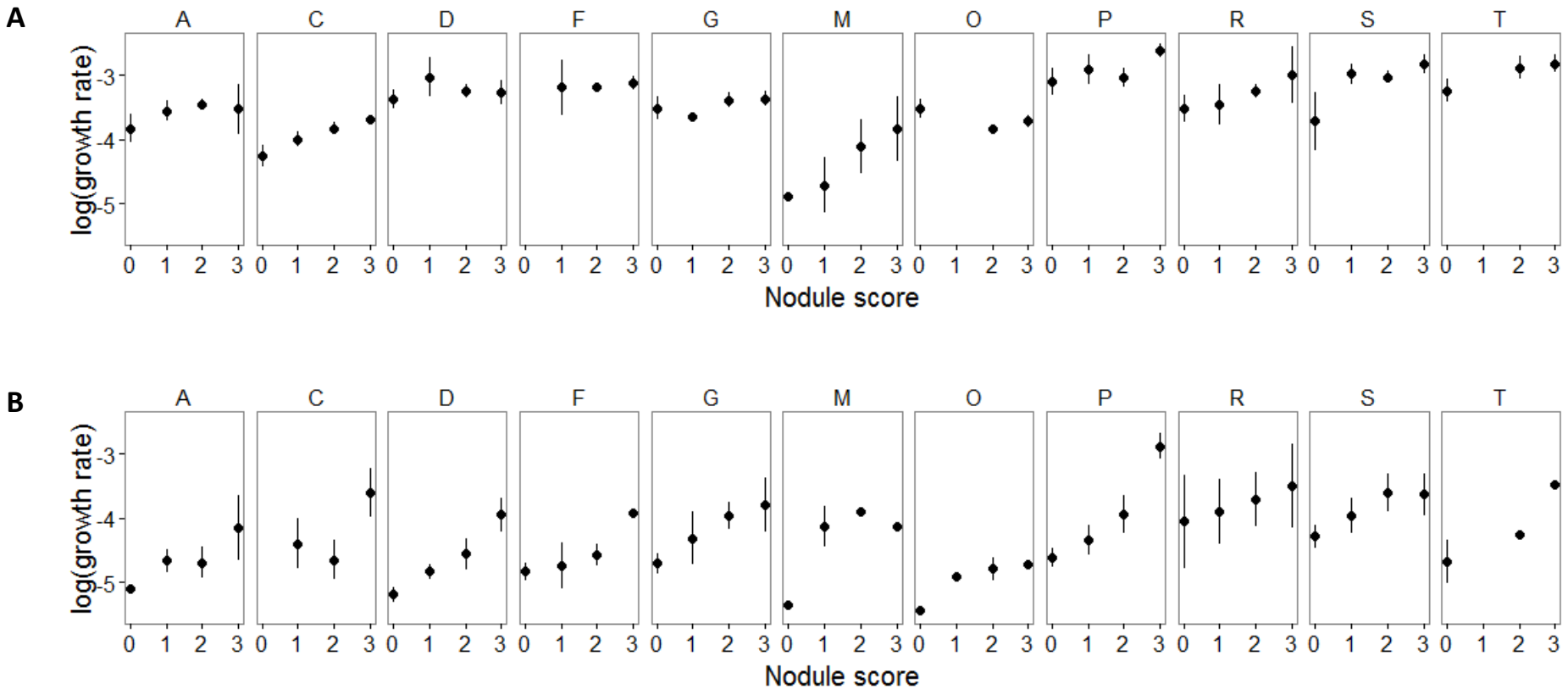

Figure H.3. Relationships between the degree of rhizobia root nodulation and plant growth rate for each *Trifolium* species in the European glasshouse (A) and in the New Zealand glasshouse (B). Points represent mean growth rates for each nodulation score; bars represent 95% confidence intervals. Plot titles indicate *Trifolium* species: A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* F = *T. fragiferum,* G = *T. glomeratum,* M = *T. micranthum,* P = *T. pratense,* R = *T. repens,* S = *T. striatum,* O = *T. ornithopodioides,* T = *T. tomentosum*.

Appendix I: Plant-soil feedback calculation approach (Chapter 3)

The plant-soil feedback responses (PSF) presented in Chapter 3 were calculated using a linear mixed model framework (see section 3.3.5 in Chapter 3). This was used to statistically remove the effect of rhizobia and account for the presence of rhizobia contaminants in sterilised treatments, but it also allowed us to focus on plant growth responses resulting from components of soil biota other than rhizobia. To verify that this approach to calculating PSF (M1) was robust, we compared the resulting PSF responses to those calculated using two additional approaches: 1) calculation of PSF without the use of a model (standard approach); and 2) using a linear mixed model that did not remove the rhizobia effect (M2).

PSF responses were calculated for each *Trifolium* species, seed origin (UK or Spain) and soil origin (NZ, UK or Spain) using the equation: $mean\left(\ln\left(\frac{growth\ rate\ in\ live\ soil}{growth\ rate\ in\ sterilised\ soil}\right)\right)$. For the standard approach, PSF was calculated in a 'pairwise' manner, whereby each PSF ratio comprised of pairs of live-sterilised soil observations that originated from the same field site, rhizobia treatment and year of experiment. In the 2013 experiments, the data included within-field site replicates, as there were two observations for each live and sterilised soil per field site for each species, seed and soil origin. These within-site replicates were dealt with in the standard approach by randomly pairing up the live and sterilised soil observations per field site for each species, seed and soil origin. For a full explanation of the model framework used to statistically remove the effect of rhizobia from PSF responses, see section 3.3.5 in Chapter 3 and model M1 in Table I.1-I.3 in this Appendix. For an explanation of the model framework used to calculate PSF without removing the rhizobia effect, see model M2 in Table I.1-I.3.

Feedback values generated via the standard approach and the model approach without removing the rhizobia effect (M2) were highly correlated (Figure I.1A). These two approaches produced precisely the same values for PSF responses (for each species, seed and soil origin) for which balanced data was available (i.e. an equal number of observations in live and sterilised soil from each field site, rhizobia treatment and year of experiment). However, the data for some PSF responses included missing observations that resulted from plants dying (46 out of 886 plants died). In the standard approach, these missing observations were dealt with by omitting the available paired observations (i.e. a dead plant in live soil results in loss of the available paired observation in sterilised soil). In contrast, the model approach uses all available data, explaining the discrepancies between the standard and M2 approach (shown as crosses on Figure I.1A). Statistically removing the effect of rhizobia from PSF responses using model M1 produced values that were generally less

positive than those calculated by M2, in line with expectations of removing the effect of a mutualist from the soil community (Figure I.1B).

Table I.1. R code for linear mixed models used to calculate plant-soil feedback (PSF). M1 was used to statistically remove the rhizobia effect from PSF responses, whereas M2 did not account for the rhizobia effect. See Table I.2 for a breakdown of the variables.

Model	R code
M1	lmer(log(growth rate) ~ group-1 + rhizo + nods + (nods-1|group) + (1|ref))
M2	lmer(log(growth rate) ~ group-1 + (1|ref))

Table I.2. Breakdown of variables included in the linear mixed models used to calculate plant-soil feedback responses according to the R code in Table I.1.

Model variable	Fixed / random effect in model	Explanation
group-1	Fixed	A categorical variable referring to live or sterilised soil for each *Trifolium* species, seed origin (UK or Spain) and soil origin (UK, Spain or New Zealand). The '-1' was included to specify a regression without a constant intercept term, so that the fixed effect coefficients were expressed as separate estimates of plant growth rates in the absence of rhizobia for each 'group' category (as opposed to the model default, which would express coefficients as the difference between the 'group' categories).
nods	Fixed	Nodulation score: a variable indicating the degree of functional nitrogen-fixation by rhizobia bacteria for each plant. This variable was included to model relationships between plant growth rate and nodulation score. The fixed effect intercept coefficients of the fitted model then represented estimates of growth rates at a nodulation score of zero, thereby statistically removing the effect or rhizobia.
rhizo	Fixed	Rhizobia treatment: a variable indicating whether a commercial rhizobia strain was added or not. Inclusion of this variable modelled relationships between plant growth rate and rhizobia addition. The fixed effect coefficients were then expressed as growth rate estimates as if commercial rhizobia was absent, in addition to zero nodulation.
(nods-1|group)	Random	This effect allowed the intercepts of the growth rate x nodulation score regressions to vary by live or sterilised soil treatments for each species, seed origin (UK or Spain) and soil origin (UK, Spain or NZ), so that growth rates in the absence of rhizobia were estimated separately for each of these 'group' categories.
(1|ref)	Random	'ref' was a categorical variable referring to groups of data from the same site of field soil collection, rhizobia treatment and year of experiment for each species, seed origin (UK or Spain) and soil origin (UK, Spain or NZ). This effect was included in order to take account of the variation in plant growth rates resulting from these variables by allowing the model intercept coefficients to vary by these groups.

Table I.3. Output statistics for linear mixed model M1 used to statistically remove the effect of rhizobia from plant-soil feedback responses.

Fixed effects				
Variable	Estimate	Std. Error	t value	P value
rhizo	0.06	0.05	1.34	0.181
nods2	0.26	0.04	6.22	<0.001

Random effects			
Variable	Variance	Std. Dev.	P value
Ref	0.04	0.21	<0.001
group:nods	0.06	0.25	0.05
Residual	0.21	0.46	

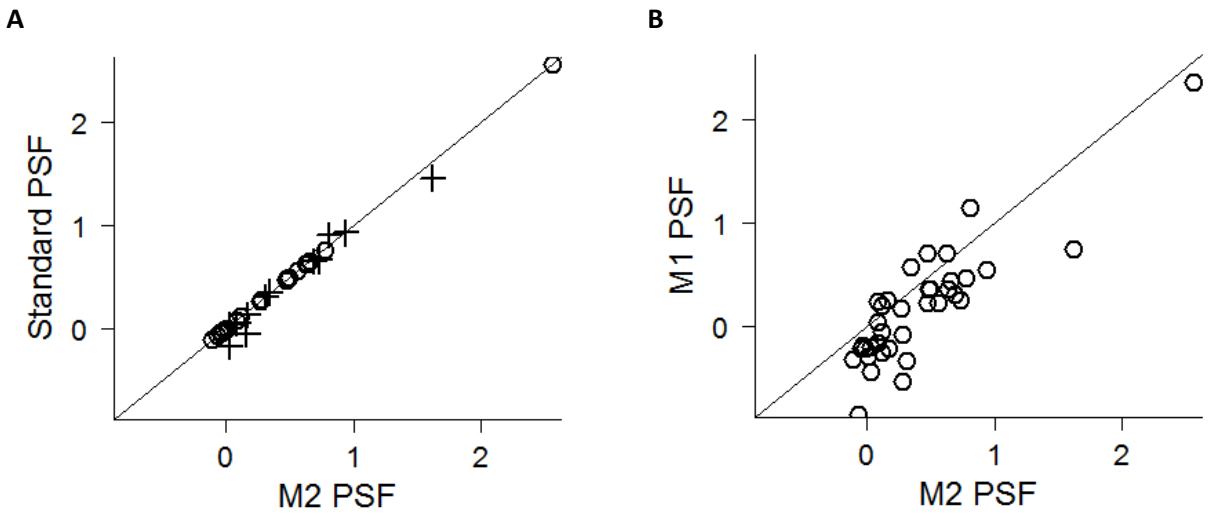

Figure I.1. Comparing plant-soil feedback (PSF) responses calculated by three different statistical approaches. A = a linear mixed model approach that did not remove the effect of rhizobia from PSF responses (M2), versus the standard non-model approach (also not removing the rhizobia effect). Crosses on plot A indicate PSF responses that had an unbalanced number of observations from live and sterilised soil per field site, rhizobia treatment and each year of experiment due to plants dying; circles indicate PSF responses that had balanced data. B = a linear mixed model approach that did not remove the effect of rhizobia from PSF responses (M2), versus a linear mixed model that did statistically remove the rhizobia effect (M1). The M1 responses were used for analyses in Chapter 3.

Appendix J: Plant-soil feedback responses separated by country of seed and soil origin (Chapter 3)

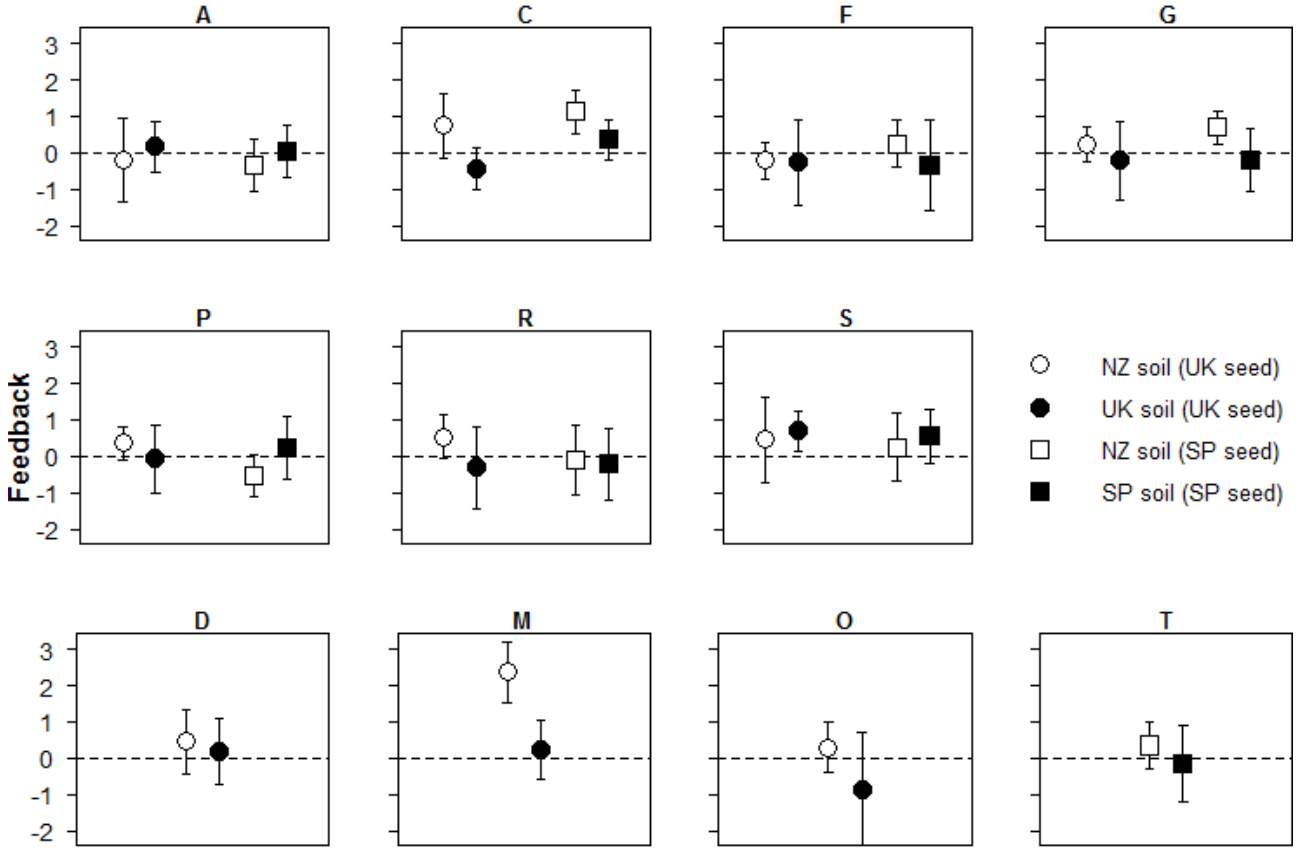

Figure J.1. Mean plant-soil feedback (PSF) responses with 95% confidence intervals for each *Trifolium* species by country of seed origin (UK/Spain) and soil origin (NZ/UK/Spain). Plot titles represent *Trifolium* species: A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* F = *T. fragiferum,* G = *T. glomeratum,* M = *T. micranthum,* P = *T. pratense,* R = *T. repens,* S = *T. striatum,* O = *T. ornithopodioides,* T = *T. tomentosum*. The dashed line represents no difference in plant growth between live and sterilised soil treatments (neutral feedback), positive feedback results from a faster growth rate in live than in sterilised soil and negative feedback results from a slower growth rate in live than sterilised soil.

Appendix K: Trends between the strength of release from inhibitory plant-soil feedback and time since naturalisation and geographic spread of the species, separately for the NZ-UK and NZ-Spain comparisons (Chapter 3)

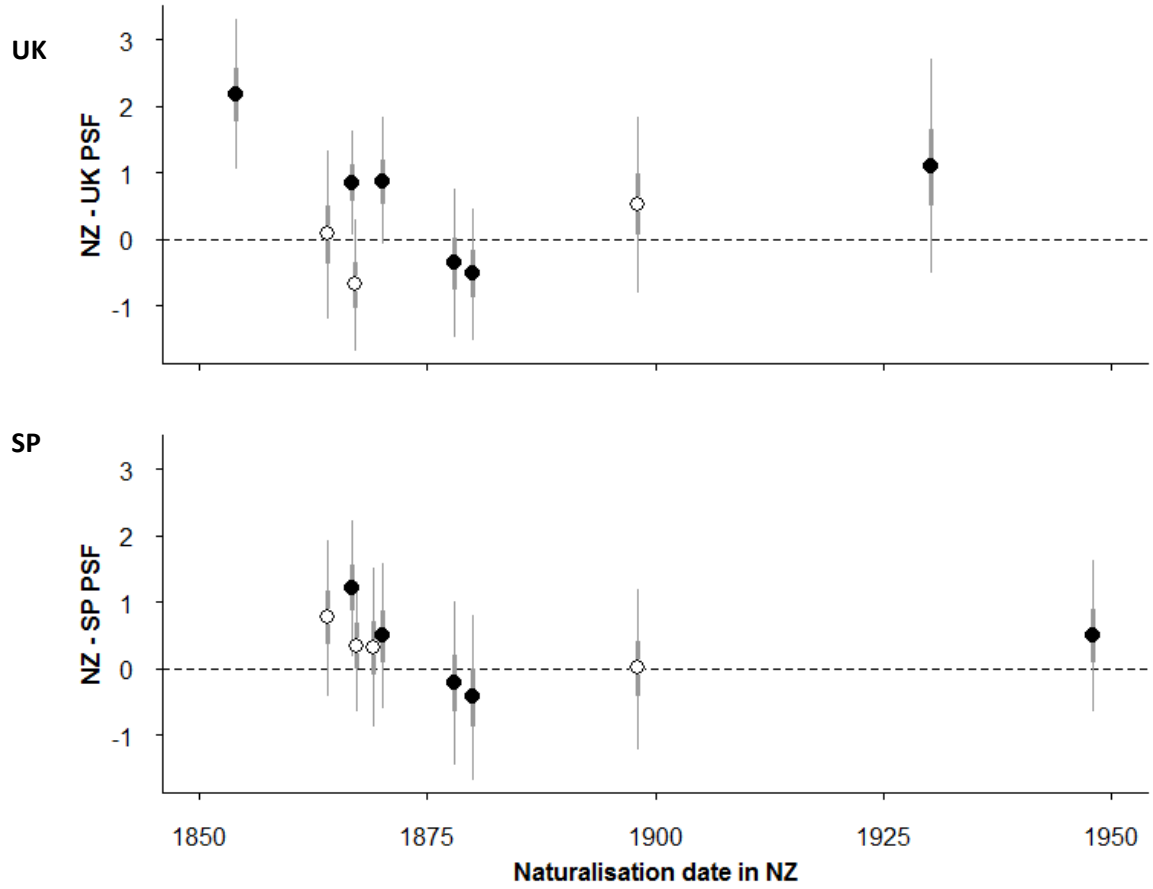

Figure K.1. Mean biogeographic differences in plant-soil feedback (PSF) for each *Trifolium* species as a function of their naturalisation dates in NZ, separately for comparisons of NZ vs. UK and NZ vs. Spain. Bars represent 95% (thin bars) and 50% (thick bars) confidence intervals. Symbols: ● = accidental introductions; ○ = intentional introductions. The dashed line represents no difference in PSF between ranges. Positive values represent more positive PSF in NZ relative to the native range, indicating a release from inhibitory soil biota, and vice-versa for negative values. *T. campestre* and *T. pratense* had identical naturalisation dates (1867) so were jittered by half a year on the x-axis.

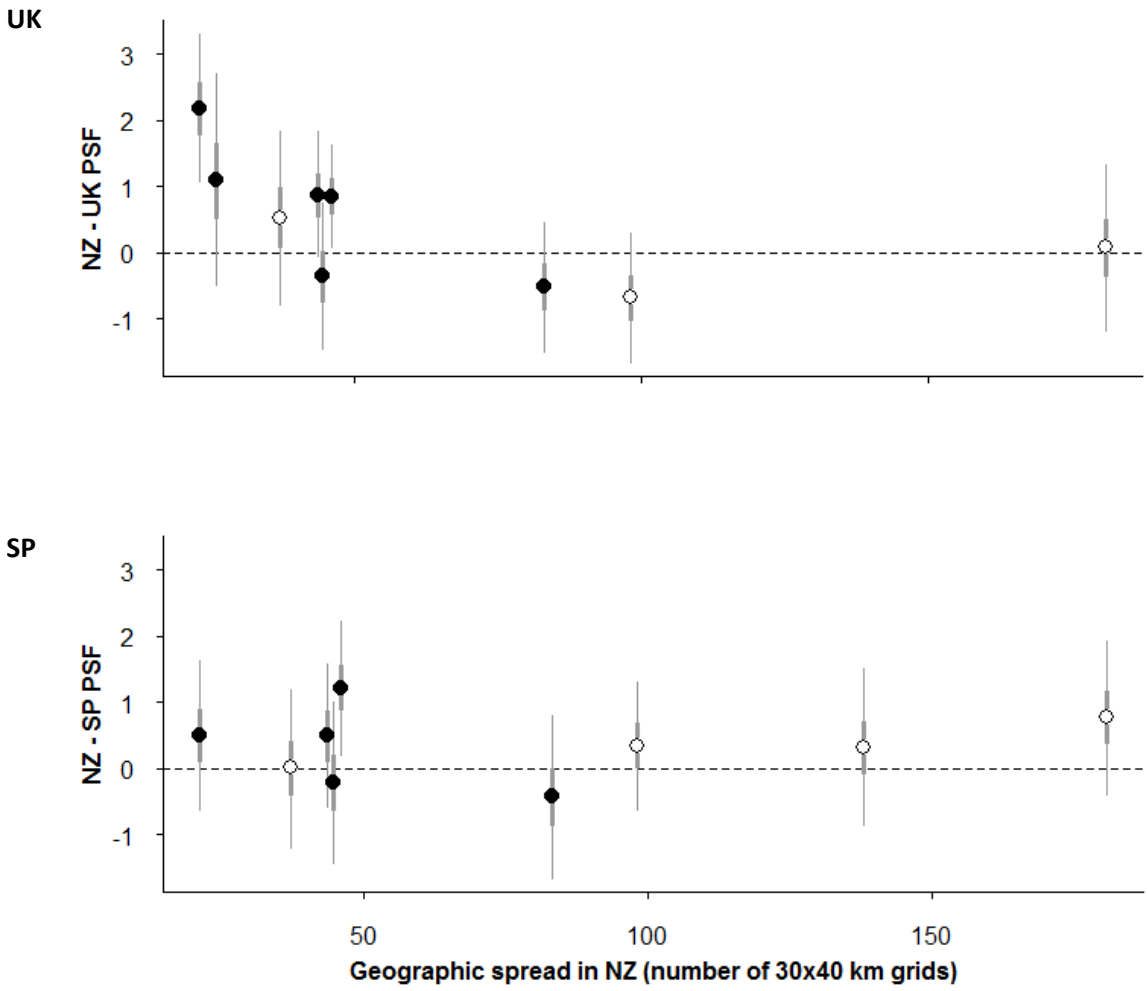

Figure K.2. Mean biogeographic differences in plant-soil feedback (PSF) for each *Trifolium* species as a function of their geographic spread NZ, separately for comparisons of NZ vs. UK and NZ vs. Spain. Bars represent 95% (thin bars) and 50% (thick bars) confidence intervals. Symbols: ● = accidental introductions; ○ = intentional introductions. The dashed line represents no difference in PSF between ranges. Positive values represent more positive PSF in NZ relative to the native range, indicating a release from inhibitory soil biota, and vice-versa for negative values. *T. glomeratum* and *T. striatum* had identical spread (44 grids) so were jittered by a value of one grid on the x-axis.

Appendix L: Supporting information for Chapter 4

Table L.1. Sampling sites for root collection in New Zealand (NZ) and the United Kingdom (UK). *Trifolium* species: A = *T. arvense*; F = *T. fragiferum*; R = *T. repens*. The final column states the number of root samples from separate plants at each site used for TRFLP analysis (63 root samples in total). The distance between sampling sites in NZ 42 km, whereas in the UK it was 198 km.

Species	Range	Site number	Location	Latitude	Longitude	Soil description	No. of root samples
A	NZ	n1	Pope Street, Christchurch	-43.536548	172.609818	Waste ground, well-drained, rocky soil	4
A	NZ	n2	Little River, Banks Peninsula	-43.769456	172.790641	Well-drained, gravelly soil	3
A	NZ	n6	Kaitorete Spit, Banks Peninsula	-43.825509	172.698964	Dunes, sandy soil	3
A	NZ	n7	Birdlings Flat, Banks Peninsula	-43.815543	172.698964	Dry grassy road verge	3
F	NZ	n11	Motukarara, Banks Peninsula	-43.729049	172.584264	Damp, organic rich loam	2
F	NZ	n12	Lake Forsyth, Banks Peninsula	-43.809746	172.723392	Marshland, silty soil	3
F	NZ	n14	Streeters Road, Banks Peninsula	-43.736169	172.625697	Gravelly, moist loam	3
R	NZ	n1	Pope Street, Christchurch	-43.536548	172.609818	Waste ground, well-drained, rocky soil	4
R	NZ	n2	Little River, Banks Peninsula	-43.769456	172.790641	Well-drained, gravelly soil	3
R	NZ	n3	Bossu Road, Banks Peninsula	-43.802360	172.841276	Grassland, clayey soil	3
R	NZ	n4	Chorlton Road, Banks Peninsula	-43.675339	173.044438	Road verge, clayey, gravelly soil	2
R	NZ	n5	Okains Bay, Banks Peninsula	-43.704496	173.047237	Road verge, well-drained, sandy soil	3
A	UK	u1	Bournemouth, England	50.719495	-1.857508	Road verge, well-drained, sandy soil	3
A	UK	u20	Pennard Burrows, Gower, Wales	51.576022	-4.091374	Dunes, organic rich sandy soil	3
A	UK	u22	Kenfig, Gower, Wales	51.515509	-3.727773	Dunes, sandy soil	3
F	UK	u25	Crofty, Gower, Wales	51.639580	-4.137559	Marshy, silty, organic rich soil	3
F	UK	u29	Pwll, Llanelli, Wales	51.684829	-4.202671	Damp grassy verge, organic rich loam	3
F	UK	u3	Stanpit Marsh, Christchurch, England	50.730127	-1.762038	Wet sandy soil	3
R	UK	u19	Port Eynon, Gower, Wales	51.543870	-4.211144	Well-drained sandy soil	3
R	UK	u26	Gorseinon, Swansea, Wales	51.673771	-4.037835	Grassland, organic rich, coal deposits	3
R	UK	u8	West Knighton, Dorset, England	50.686878	-2.390506	Hedgerow, clay soil	3

Table L.2. Number of plant root samples and field sites used for each *Trifolium* species (A = *T. arvense*; F = *T. fragiferum*; R = *T. repens*) in New Zealand (NZ) and the United Kingdom (UK).

Species	Range	Number of samples	Number of sites
A	NZ	13	4
A	UK	9	3
F	NZ	8	3
F	UK	9	3
R	NZ	15	5
R	UK	9	3

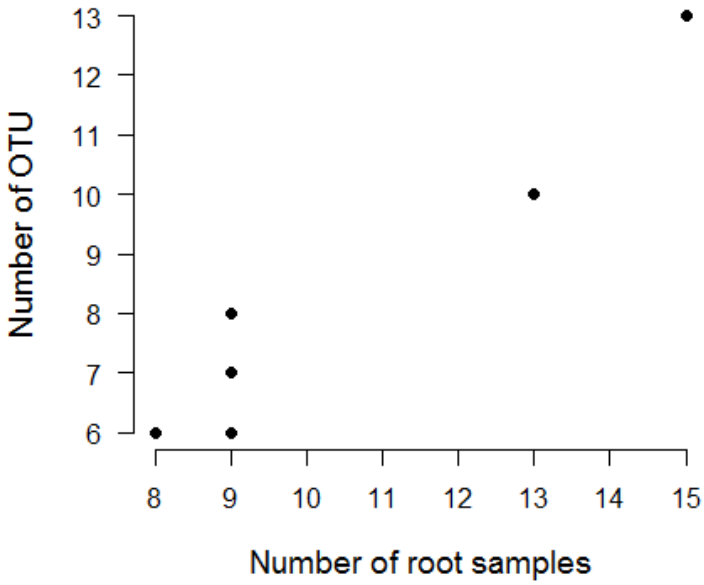

Figure L.1. Number of arbuscular mycorrhizal operational taxonomic units (OTU) detected as a function of the number of root samples used per *Trifolium* species in each range.

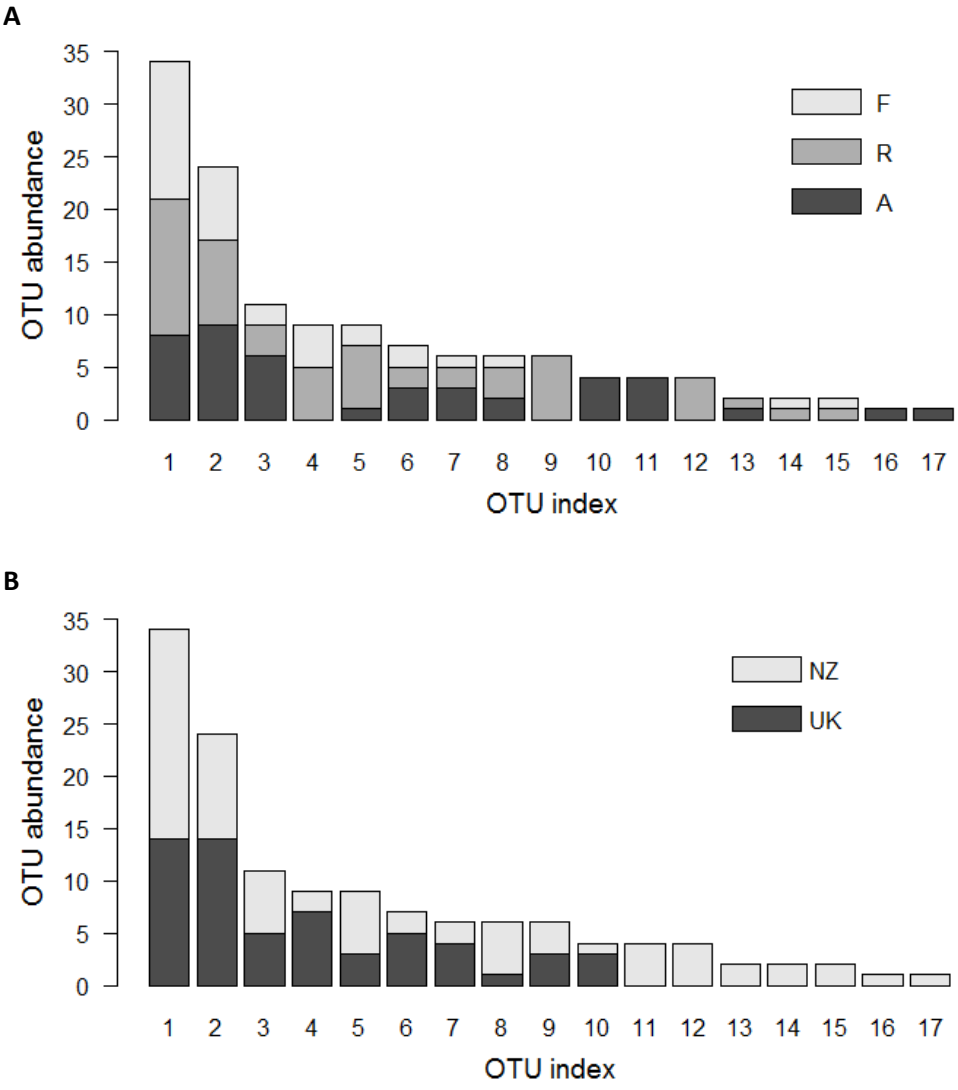

Figure L.2. Number of root samples in which each arbuscular mycorrhizal fungal operational taxonomic unit (OTU) was detected, by: A) *Trifolium* host species (F = *T. fragiferum*, R = *T. repens*, A = *T. arvense*); and B) geographic range.

Appendix M: Field site and seed source details (Chapter 5)

Table M.1. Field sites at which root nodules were sampled for each *Trifolium* species (A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum*) in New Zealand (NZ) and the United Kingdom (UK).

Species	Country	Site no.	Locality	Latitude	Longitude
A	UK	1	Bournemouth, 'Monkey Island'	50.719495	-1.857508
A	UK	2	Gosport - Browndown	50.792363	-1.193203
A	UK	3	Devon - Off Ilsham Marine Drive, near Torquay	50.458359	-3.491381
A	UK	4	Swansea marina	51.614266	-3.930945
A	UK	5	Kenfig	51.515509	-3.727773
C	UK	1	Gosport - Browndown	50.793469	-1.194668
C	UK	2	Gower - near Whiteford Burrows	51.619075	-4.255334
C	UK	3	Devon - near Chudleigh	50.614183	-3.623614
C	UK	4	Devon - headland south of Berryhead	50.382074	-3.499986
C	UK	5	Kenfig - near Sharkham Point	51.506086	-3.743090
D	UK	1	West Knighton	50.686890	-2.390173
D	UK	2	Bournemouth	50.720274	-1.849621
D	UK	3	Devon - near Chudleigh	50.614117	-3.623618
D	UK	4	Gower - Port Eynon	51.544036	-4.211099
D	UK	5	Gower - Crofty	51.639117	-4.136976
O	UK	1	Bournemouth	50.720299	-1.849496
O	UK	2	Gosport	50.792120	-1.180596
O	UK	3	Gower - Port Eynon	51.543854	-4.211267
O	UK	4	Gower - Loughour Estuary	51.630818	-4.134735
O	UK	5	Kenfig - old road near Sharkham point	51.506815	-3.740627
P	UK	1	West Knighton roadside bank	50.687200	-2.381096
P	UK	2	Devon - Berryhead	50.394077	-3.492447
P	UK	3	Gower - Pennard Burrows	51.577112	-4.090455
P	UK	4	Kenfig	51.515440	-3.727951
P	UK	5	Gower - Crofty	51.638975	-4.136470
R	UK	1	West Knighton	50.686878	-2.390506
R	UK	2	Bournemouth	50.720274	-1.849621
R	UK	3	Devon - near Chudleigh	50.614043	-3.623618
R	UK	4	Gower - Port Eynon	51.543870	-4.211144
R	UK	5	Swansea - Gorseinon park	51.673771	-4.037835
S	UK	1	Bournemouth, 'Monkey Island'	50.719368	-1.858172
S	UK	2	Gosport - Browndown	50.793469	-1.194668
S	UK	3	Devon - headland south of Berryhead	50.382116	-3.499871
S	UK	4	Gower - Broadpool	51.597861	-4.151891
S	UK	5	Gower - Pennard Burrows	51.575920	-4.091192
A	NZ	1	Pope Street, Addington	172.609618	-43.536464
A	NZ	2	Kaitorete Spit	172.698799	-43.701749
A	NZ	3	HW 1 Bridge	172.232281	-43.646740
A	NZ	4	Wakanui Beach Road, Wakanui	171.863984	-44.026936
A	NZ	5	Seadown Rd, Timaru	171.286188	-44.265423
C	NZ	1	Streeters Road	172.625468	-43.736258
C	NZ	2	Western Valley Road	172.795569	-43.747027
C	NZ	3	Big Hill Road, Banks Peninsula	173.064849	-43.701749

C	NZ	4	Wakanui School Road, Wakanui	171.814292	-43.976931
C	NZ	5	Seadown Rd, Timaru	171.284508	-44.270326
D	NZ	1	Bridleway, Governors Bay	172.651952	-43.632160
D	NZ	2	Lake Forsyth	172.723295	-43.809611
D	NZ	3	HW 1 Bridge	172.231962	-43.646715
D	NZ	4	Wakanui Beach Road, Wakanui	171.864058	-44.026713
D	NZ	5	Caroline Bay, Timaru	171.251068	-44.389271
P	NZ	1	Roadside, Governors Bay Teddington Road, Teddington	172.655803	-43.651580
P	NZ	2	Days Road, Springston	172.437424	-43.645590
P	NZ	3	McCrorys Road, Pendarves	171.987335	-43.885801
P	NZ	4	Waipopo Road, Seadown	171.290495	-44.288008
P	NZ	5	Withells Rd, Ealing	171.417599	-44.044594
R	NZ	1	Pope Street, Addington	172.609757	-43.536279
R	NZ	2	Chorlton Road, Okains Bay, Banks Peninsula	173.047200	-43.704497
R	NZ	3	Corbetts Road, Wakanui	171.895358	-44.001981
R	NZ	4	Caroline Bay, Timaru	171.250939	-44.389192
R	NZ	5	Seadown Rd, Seadown	171.284746	-44.269265
S	NZ	1	Heathcote Quarry Track at Major Hornbrook Road, Mount Pleasant	172.717020	-43.571704
S	NZ	2	Wakanui Beach road	171.864023	-44.026671
S	NZ	3	Western Valley Road	172.795645	-43.747033
S	NZ	4	Big Hill Road, Banks Peninsula	173.064903	-43.701631
S	NZ	5	Shaw Street/Domain Ave, Temuka	171.295862	-44.249368
O	NZ	1	Roadside, Governors Bay Teddington Road, Teddington	172.656328	-43.651840
O	NZ	2	Lincoln University campus	172.468438	-43.643517
O	NZ	3	Lake Forsyth	172.723000	-43.809847
O	NZ	4	Caroline Bay, Timaru	171.251316	-44.389350
O	NZ	5	Nancy Ave, Mariehau	172.638089	-43.500066

Table M.2. Seed sources from NZ and the UK used for each *Trifolium* species (A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum*). AgR = Margot Forde Germplasm Centre, AgResearch, Palmerston North, New Zealand (AgR); HS = Herbiseed, Twyford, England; Aber = Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales.

Species	Provenance	Source/accession	Specific origin	Latitude	Longitude
A	NZ	AgR (1642)	McKenzie Basin, Canterbury	NA	NA
C	NZ	AgR (2597)	Opua, Auckland	NA	NA
D	NZ	Hand collected	Kaitorete Spit, View Hill Road, Banks Peninsula	-43.825509	172.698964
P	NZ	AgR (2370)	Ngunguru, Auckland	NA	NA
R	NZ	Hand collected	Streeters Road, Banks Peninsula	-43.734425	172.625366
S	NZ	Hand collected	Bridleway, Governors Bay	-43.632091	172.652055
O	NZ	Hand collected	Squally Bay, Banks Peninsula	-43.893924	172.914841
A	UK	Hand collected	Pennard Burrows, Gower	51.576022	-4.091374
C	UK	Hand collected	Crymlyn, Swansea	51.624223	-3.843038
D	UK	Hand collected	Marina, Swansea	51.614266	-3.930945
P	UK	Herbiseed	NA	NA	NA
R	UK	Hand collected	Pennard Burrows, Gower	51.576022	-4.091374
S	UK	Hand collected	Bournemouth	50.720274	-1.849621
O	UK	Aber (1688-1984U)	Somerset	NA	NA

Appendix N: Lab method details (Chapter 5)

Table N.1. Yeast mannitol agar composition (Vincent 1970).

Ingredient	Grams/Litre DI H_20
Yeast extract	1
Mannitol	10
K_2HPO_4 (dipotassium hydrogen phosphate)	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	1
Agar	15

Table N.2. Yeast mannitol broth composition (Vincent 1970).

Ingredient	Grams/Litre DI H_20
Yeast extract	1
Mannitol	10
Dipotassium phosphate (dipotassium hydrogen phosphate)	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Calcium carbonate	1

Table N.3. Primer nucleotide sequences.

Primer	Sequence (from 5' to 3')	Amplification	Reference
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	Repetitive intergenic consensus sequences	(Versalovic *et al.* 1994)
ERIC2	AAGTAAGTGACTGGGGTGAGCG	Repetitive intergenic consensus sequences	(Versalovic *et al.* 1994)
Y5	ATGCGKTTYARRGGMCTNGATCT	nodD	(Zézé *et al.* 2001)
Y6	CGCAWCCANATRTTYCCNGGRTC	nodD	(Zézé *et al.* 2001)
27f	AGAGTTTGATCMTGGCTCAG	Universal bacterial primer	(Lane 1991)
1492r	GRTACCTTGTTACGACTT	Universal bacterial primer	(Lane 1991)

Table N.4. Phosphate peptone buffer composition (Prévost & Antoun 2007).

Ingredient	Grams/Litre DI H_20
Peptone	1.0 g
KH_2PO_4 (potassium di-hydrogen phosphate)	0.34 g
K_2HPO_4 (dipotassium hydrogen phosphate)	1.21 g

Appendix O: Isolates used for molecular analyses (Chapter 5)

Table O.1. Rhizobia isolates used for rep-PCR fingerprinting for each *Trifolium* species (A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* P = *T. pratense,* S = *T. striatum*), in each range (New Zealand or United Kingdom), with field site numbers according to Table M.1 in Appendix M.

Species	Range/field site	Number of isolates (from individual nodules from separate plants)
A	NZ1	3
A	NZ2	2
A	NZ3	2
A	NZ5	3
A	UK1	3
A	UK2	1
A	UK3	3
A	UK4	2
C	NZ1	1
C	NZ2	3
C	NZ3	1
C	NZ4	3
C	NZ5	3
C	UK1	3
C	UK2	3
C	UK3	3
C	UK4	2
C	UK5	2
D	NZ1	1
D	NZ2	3
D	NZ4	2
D	NZ5	1
D	UK1	3
D	UK2	2
D	UK3	3
D	UK4	3
D	UK5	2
P	NZ1	3
P	NZ2	3
P	NZ3	3
P	NZ4	1
P	UK1	2
P	UK2	3
P	UK3	3
P	UK4	3
P	UK5	2
S	NZ1	2
S	NZ3	3
S	NZ4	3
S	NZ5	1
S	UK1	3
S	UK2	3
S	UK3	2
S	UK4	3
S	UK5	2

Table O.2. Rhizobia isolates used for the nodD phylogeny for each *Trifolium* species (A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum*) from each range (New Zealand or United Kingdom), with field site numbers according to Table M.1 in Appendix M and the plant number from that site.

Isolate reference	Species	Range/field site	Plant number
a11n	A	NZ1	1
a11u	A	UK1	1
a12u	A	UK1	2
a13n	A	NZ1	3
a13u	A	UK1	3
a21n	A	NZ2	1
a22u	A	UK2	2
a31u	A	UK3	1
a32n	A	NZ3	2
a33u	A	UK3	3
a41u	A	UK4	1
a42u	A	UK4	2
a51n	A	NZ5	1
a52n	A	NZ5	2
a52u	A	UK5	2
a53n	A	NZ5	3
c11u	C	UK1	1
c12u	C	UK1	2
c13n	C	NZ1	3
c13u	C	UK1	3
c21n	C	NZ2	1
c21u	C	UK2	1
c22n	C	NZ2	2
c22u	C	UK2	2
c23n	C	NZ2	3
c31n	C	NZ3	1
c31u	C	UK3	1
c32n	C	NZ3	2
c32u	C	UK3	2
c33n	C	NZ3	3
c33u	C	UK3	3
c41n	C	NZ4	1
c41u	C	UK4	1
c42u	C	UK4	2
c43n	C	NZ4	3
c43u	C	UK4	3
c51n	C	NZ5	1
c51u	C	UK5	1
c52n	C	NZ5	2
c52u	C	UK5	2
c53n	C	NZ5	3
d11u	D	UK1	1
d12u	D	UK1	2
d13n	D	NZ1	3
d13u	D	UK1	3
d21n	D	NZ2	1
d21u	D	UK2	1

d23n	D	NZ2	3
d23u	D	UK2	3
d31u	D	UK3	1
d32u	D	UK3	2
d33u	D	UK3	3
d41u	D	UK4	1
d42n	D	NZ4	2
d43u	D	UK4	3
d51u	D	UK5	1
d53n	D	NZ5	3
o11n	O	NZ1	1
o11u	O	UK1	1
o12u	O	UK1	2
o13u	O	UK1	3
o21n	O	NZ2	1
o21u	O	UK2	1
o22n	O	NZ2	2
o23u	O	UK2	3
o31n	O	NZ3	1
o31u	O	UK3	1
o32n	O	NZ3	2
o32u	O	UK3	2
o33n	O	NZ3	3
o33u	O	UK3	3
o41u	O	UK4	1
o42n	O	NZ4	2
o42u	O	UK4	2
o43n	O	NZ4	3
o52u	O	UK5	2
o53u	O	UK5	3
p11u	P	UK1	1
p12n	P	NZ1	2
p12u	P	UK1	2
p13n	P	NZ1	3
p13u	P	UK1	3
p21u	P	UK2	1
p22n	P	NZ2	2
p22u	P	UK2	2
p23n	P	NZ2	3
p23u	P	UK2	3
p31n	P	NZ3	1
p31u	P	UK3	1
p32n	P	NZ3	2
p32u	P	UK3	2
p33n	P	NZ3	3
p33u	P	UK3	3
p41u	P	UK4	1
p42n	P	NZ4	2
p42u	P	UK4	2
p43u	P	UK4	3
p51u	P	UK5	1
p53u	P	UK5	3
r11u	R	UK1	1
r12u	R	UK1	2

r13u	R	UK1	3
r21n	R	NZ2	1
r21u	R	UK2	1
r23n	R	NZ2	3
r23u	R	UK2	3
r31n	R	NZ3	1
r31u	R	UK3	1
r32n	R	NZ3	2
r33u	R	UK3	3
r41n	R	NZ4	1
r41u	R	UK4	1
r42n	R	NZ4	2
r42u	R	UK4	2
r43n	R	NZ4	3
r52n	R	NZ5	2
r52u	R	UK5	2
r53u	R	UK5	3
s11n	S	NZ1	1
s13n	S	NZ1	3
s22u	S	UK2	2
s32n	S	NZ3	2
s32u	S	UK3	2
s41n	S	NZ4	1
s41u	S	UK4	1
s42n	S	NZ4	2
s42u	S	UK4	2
s43n	S	NZ4	3
s43u	S	UK4	3
s51u	S	UK5	1
s53u	S	UK5	3

Appendix P: Statistical output tables (Chapter 5)

Table P.1. Output from a linear mixed model that tested whether nodulation score (degree of root nodulation by rhizobia), rhizobia provenance (rhizo = New Zealand or United Kingdom), seed provenance (seed = New Zealand or United Kingdom), and an interaction between rhizobia and seed provenance (rhizo:seed) significantly influenced plant growth rates.

Fixed effects				
	Estimate	**Std. Error**	**t value**	**p value**
Intercept	-4.15	0.16	-25.3	
nodulation	0.08	0.03	3.2	0.001
rhizo	0.01	0.04	0.2	0.820
seed	-0.06	0.04	-1.4	0.158
rhizo:seed	-0.02	0.06	-0.3	0.765

Random effects		
	Variance	**Std. Dev.**
species	0.17	0.42
residual	0.05	0.23

Table P.2. Output from linear models that tested whether rhizobia provenance (rhizo = New Zealand or United Kingdom), seed provenance (seed = New Zealand or United Kingdom) and an interaction between the two variables (rhizo:seed) significantly influenced growth rates of each *Trifolium* species.

	Estimate	Standard error	t value	p value
T. arvense				
Intercept	-4.15	0.06	-67.85	
rhizo	-0.11	0.09	-1.26	0.22
seed	-0.31	0.09	-3.59	< 0.001
rhizo:seed	-0.08	0.12	-0.62	0.54
T. campestre				
Intercept	-4.40	0.05	-85.12	
rhizo	0.02	0.07	0.23	0.82
seed	-0.46	0.07	-6.30	< 0.001
rhizo:seed	0.02	0.10	0.23	0.82
T. dubium				
Intercept	-4.25	0.07	-59.99	
rhizo	0.01	0.10	0.12	0.90
seed	0.41	0.10	4.05	< 0.001
rhizo:seed	-0.15	0.14	-1.03	0.31
T. ornithopodioides				
Intercept	-4.42	0.06	-68.02	
rhizo	0.06	0.09	0.61	0.54
seed	0.15	0.09	1.61	0.12
rhizo:seed	-0.19	0.13	-1.46	0.15
T. pratense				
Intercept	-3.54	0.07	-51.64	
rhizo	0.01	0.10	0.08	0.94
seed	0.18	0.10	1.81	0.08
rhizo:seed	-0.08	0.14	-0.55	0.59
T. repens				
Intercept	-3.66	0.07	-54.58	
rhizo	0.01	0.09	0.06	0.95
seed	-0.16	0.09	-1.67	0.10
rhizo:seed	0.22	0.13	1.68	0.10
T. striatum				
Intercept	-3.84	0.06	-68.83	
rhizo	0.11	0.08	1.35	0.19
seed	0.00	0.08	-0.04	0.97
rhizo:seed	-0.07	0.11	-0.63	0.54

Appendix Q: Nodulation x growth rate relationships (Chapter 5)

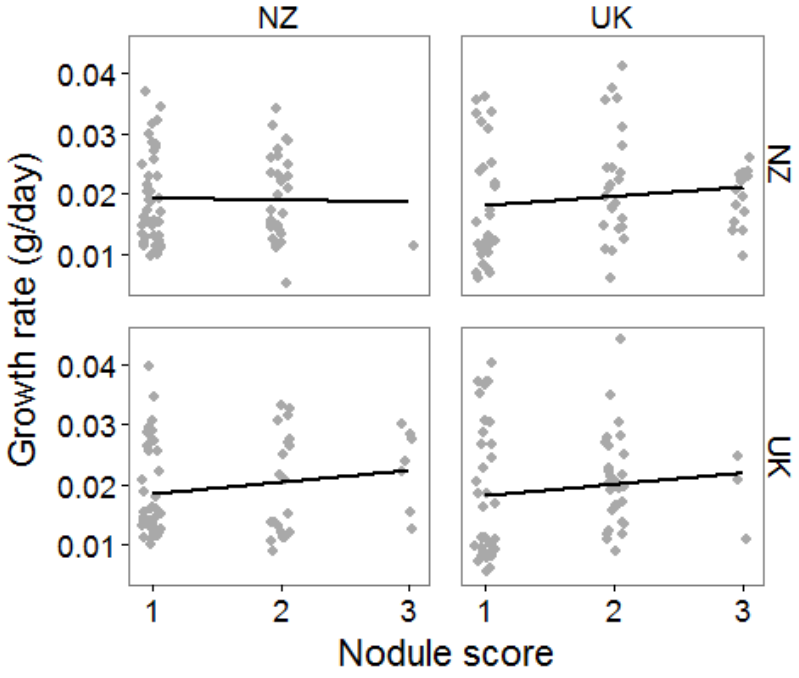

Figure Q.1. Relationships between plant growth rate (grams of dry biomass per glasshouse-grown day) and rhizobia nodulation score by seed range (horizontal panels) and rhizobia range (vertical panels), with linear regressions. Points have been jittered around nodulation scores on the x axes.

Appendix R: Glasshouse inocula details (Chapter 5)

Table R.1. Number of isolates comprising each bulk bacterial inocula used in the glasshouse experiment that were verified as rhizobia, for each range (NZ and UK) and *Trifolium* species (A = *T. arvense,* C = *T. campestre,* D = *T. dubium,* O = *T. ornithopodioides,* P = *T. pratense,* R = *T. repens,* S = *T. striatum*).

Trifolium species	Range	Number of isolates verified as rhizobia	Total number of isolates in inocula	Amount of inocula consisting of verified rhizobia (%)
A	UK	11	13	84.6
A	NZ	10	13	76.9
C	UK	12	14	85.7
C	NZ	12	14	85.7
D	UK	14	15	93.3
D	NZ	8	15	53.3
O	UK	14	14	100
O	NZ	9	14	64.3
P	UK	15	15	100
P	NZ	14	15	93.3
R	UK	12	14	85.7
R	NZ	9	14	64.3
S	UK	12	14	85.7
S	NZ	8	14	57.1

Appendix S: Blast search results (Chapter 5)

Table S.1. Genbank BLAST search results, showing the closest taxonomic matches to the 16S sequences from bacterial isolates that failed to amplify with rhizobia-specific nodD primers.

Isolate reference from this study	Genbank accession number for closest match	Taxa of closest Genbank match	Sequence similarity (%)	Overlap in sequences (%)
a23n	KJ152098.1	*Enterobacter* sp.	100	93
a42n	AB681415.1	*Bacillus psychrosaccharolyticus*	99	99
a43n	EU853188.1	*Pseudomonas* sp.	99	100
c11n	KC243291.1	*Arthrobacter* sp.	100	100
d12n	AB495351.1	*Duganella* sp.	99	100
d31n	HG940537.1	*Pseudomonas* sp.	99	99
d32n	JN853238.1	*Pantoea* sp.	93	100
d33n	JX035940.1	*Pseudomonas* sp.	99	100
d43n	AY660698.1	*Bacillus* sp.	99	99
d51n	EU853188.1	*Pseudomonas* sp.	100	100
o12n	HQ224646.1	*Erwinia billingiae*	99	99
o23n	KF749031.1	*Rhizobium leguminosarum* bv. *trifolii*	100	100
o51n	KF555635.1/ JQ782497.1	*Pseudomonas* sp. / *Pseudomonas fluorescens*	100/100	100/100
o53n	KF941208.1	*Pantoea agglomerans*	99	100
p41n	KF817809.1	*Rahnella aquatilis*	99	100
p43n	KF555635.1/ JQ782497.1	*Pseudomonas* sp. / *Pseudomonas fluorescens*	100/99	100/100
p51n	FN397515.1	*Bacillus simplex*	100	99
p52n	HM099662.1	*Erwinia* sp.	100	100
p53n	AY660698.1	*Bacillus* sp. / *Bacillus psychrosaccharolyticus*	98/98	100/100
r11n	FJ225193.1	*Pseudomonas* sp.	100	100
r13n	JQ977301.1	*Arthrobacter* sp.	99	98
r51n	KJ184943.1	*Bacillus* sp.	100	100
r53n	JN853218.1	*Pantoea* sp.	99	100
s12n	FR799426.1/ EU810854.1	*Bacillus* sp. / *Actinobacterium*	93/93	99/99
s21n	JQ977536.1	*Yonghaparkia*	99	99
s22n	JX195144.1	*Paenibacillus* sp.	99	100
s23n	JQ080603.1	*Pseudomonas* sp.	99	100
s41n	KF748994.1	*Rhizobium leguminosarum* bv. *trifolii*	100	100
s52n	HE716932.1/ NR_102966.1	*Pantoea agglomerans* / *Pantoea vagans*	94/94	100/100
s53n	GU188915.1	*Erwinia billingiae*	99	100
a21u	KC236770.1	*Paenibacillus* sp.	99	99
a23u	KF748994.1	*Rhizobium leguminosarum* bv. *trifolii*	100	100
a43u	KF748994.1	*Rhizobium leguminosarum* bv. *trifolii*	100	100
a53u	KC236770.1	*Paenibacillus* sp.	99	99
c53u	AJ315076.1	*Paenibacillus* sp.	99	99
d52u	HM566574.1	*Bacillus* sp.	92	100
r51u	AB576894.1	*Paenibacillus* sp.	99	100
s52u	JX266407.1	*Pseudomonas* sp.	99	100