Temporal dynamics and function of root-associated fungi during a non-native plant invasion

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Abstract

TEMPORAL DYNAMICS AND FUNCTION OF ROOT-ASSOCIATED FUNGI DURING A NON-NATIVE PLANT INVASION

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Net effects of root-associated fungal communities on plant growth range from positive to negative due to the combined effects of mutualists and pathogens, and are called plant-soil feedbacks. Exotic invasive plants may benefit more from associating with particular mutualists than with pathogens, resulting in overall positive feedback. Little is known about the identities and functions of root-associated fungal taxa and the time scales over which these communities may change during invasion. The overall aim of this thesis was to investigate temporal dynamics and function of root-associated fungal communities on the invasive plant, *Vincetoxicum rossicum* (Apocynaceae). A glasshouse study combined with molecular methods showed that *V. rossicum* was rapidly colonised by many mutualistic arbuscular mycorrhizal (AM) fungal taxa. However, my data suggested that it may take longer than one growing season for this species to exert major changes to the AM fungal community and associations with particular AM fungi can occur in localised areas. A second study using multiple sites representing a timeline over decades of invasion also showed no detectable pattern in total, AM, or pathogenic fungi. A plant-soil feedback study combined with high throughput sequencing showed that *V. rossicum* forms associations with a broad range of soil fungi that benefit plant growth. Large numbers of fungi that are pathogens on other plant species were detected in the roots but there was no evidence for stronger negative feedbacks or pathogen accumulation at sites of older invasion age. In a third study, plant pathogenicity tests with seven root-associated fungal taxa showed that these fungi had either neutral or positive effects on *V. rossicum* growth compared to uninoculated controls. Further testing of three of these fungi showed neutral effects on the native species *Asclepias syriaca* (Apocynaceae), but reduced the growth of *Solidago canadensis* (Asteraceae). These results suggest that in nature *V. rossicum* may gain a net benefit from all soil biota in its invasive range and may contribute to *V. rossicum*'s invasion success.

Dedication

I'd like to dedicate this thesis to the deceased and survivors of the Christchurch earthquake, New Zealand 21 February 2011. I genuinely think of you every day. Your strength through the challenges that you continue to face on a daily basis has often helped me to put my PhD work into perspective. Etu Kahikatea! (and kia kaha).

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Arohanui

nga whanau

nga whakapapa

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Chapter 1. General introduction

Exotic invasive plants dominate communities and can cause detrimental ecological, environmental and socio-economic impacts (Mack et al., 2000; Pimentel et al., 2005; Simberloff et al., 2013). Introductions of biota to new ranges can be either purposeful or accidental and the high rates of introductions are facilitated by widespread and global anthropogenic movement (Mack et al., 2000; Catford et al., 2009). Not all exotic plants go on to become invasive, but by definition invasive plants have negative impacts on the environment or soci-economic attributes of the areas they invade and whether the plant is considered invasive or not is dependent on the context and environment (Mack et al., 2000; Catford et al., 2009; Simberloff et al., 2013). Native plants can also become invasive (Catford et al., 2009; Davis et al., 2011; Simberloff et al., 2013). However, this thesis explores theories and hypotheses pertaining to invasions by exotic invasive plants (hereafter invasive plants). The ecological and environmental impact of invasive plants is a widespread conservation concern, and determining which species may become invasive and under which conditions that arises remains elusive (MacDougall et al., 2009; Pyšek et al., 2009; Gurevitch et al., 2011; Petitpierre et al., 2012; Kuester et al., 2014). Introduced species go through a series of phases before being considered invasive, summarised by Catford et al. (2009) as: transport phase, introduction to novel range, colonisation of novel range, survival and reproduction in the novel range, and finally range expansion. Success at each phase is dependent on overcoming unique sets of biotic and abiotic barriers to growth and fitness, which is why ultimately only a few exotic introductions go on to become invasive (Williamson and Fitter, 1996; Catford et al., 2009). For example, leaving behind co-evolved enemies during the transport phase is considered one important mechanism that may enable invasive plants to grow and reproduce to become dominant in new ranges (Keane and Crawley, 2002; Colautti et al., 2004; Torchin and Mitchell, 2004).

Plants interact with a wide array of soil organisms and these interactions have been shown to be important for their growth, competitive interactions and fitness (van der Putten et al., 2001; Klironomos, 2002). The importance of soil biota for determining invasion success,

facilitating subsequent spread, and impacting native communities is increasingly recognised (Richardson et al., 2000; Klironomos, 2002; Dickie et al., 2004; Reinhart and Callaway, 2004; Mangla et al., 2008; Barto et al., 2011; Callaway et al., 2011; Koch et al., 2011; Li et al., 2014; Maron et al., 2014; Pringle et al., 2009). Plants alter soil microbial communities and, in turn, these alterations can feedback to influence plant growth, termed plant-soil feedback (Bever, 1994; Bever et al., 2012). Positive plant-soil feedback occurs when the growth and/or fitness of a particular plant species increases in its own “trained” soil, and is considered to result from accumulation and recruitment of host-specific mutualists (Klironomos, 2002; Vandenkoornhuyse et al., 2002; Bever et al., 2012). In contrast, negative plant-soil feedback result in declines in growth and/or fitness, and is due to accumulation of host-specific pathogens and/or parasites (van der Putten et al., 1993; Klironomos, 2002; van de Voorde et al., 2012). Plant-soil feedbacks have the potential to alter plant community composition and structure and may even drive succession (van der Putten et al., 1993; Klironomos, 2002; Dickie et al., 2014; Flory and Bauer, 2014). Positive feedbacks are thought to reduce plant diversity, because one plant species will benefit from enhanced mutualisms and outcompete other plant species (Bever et al., 2012). In contrast, negative feedbacks are thought to increase plant diversity in communities because they prevent any one species from becoming dominant (Bever et al., 2012). These soil microbial communities can also exert long-lasting impacts on biogeochemical cycling and influence future restoration, even if eradication of the invasive plant is successful (Ehrenfeld, 2003; Belnap et al., 2005; Dickie et al., 2014; Wright et al., 2014).

Soil fungi are important for plant community structure because they can form both mutualistic and pathogenic associations with plants (Agrios, 2005; James et al., 2006; Schulz and Boyle, 2006; Smith and Read, 2008). Mycorrhizal fungi are arguably the most important mutualistic fungi. They are obligate biotrophs that form associations with up to 80% plant species and 92% families (Wang and Qiu, 2006; Smith and Read, 2008). Arbuscular mycorrhizal (AM) fungi (i.e., Phylum Glomeromycota) are the most widespread and abundant form (Wang and Qiu, 2006; Smith and Read, 2008). These fungi colonise the plant root and extend their hyphae into the soil matrix forming what is called a mycorrhiza. The plant host provides carbon from photosynthates to the fungus and the fungus transfers macro- and micronutrients from the soil, such as phosphorus. It can also provide other benefits, such as pathogen protection or increasing water uptake (Borowicz, 2001; Smith and Read, 2008; Lewandowski et al., 2013). The AM fungal symbiosis is considered to be beneficial and the level

of benefit appears to be host-fungus specific, but may also vary with time and along environmental gradients (Johnson et al., 1997; Hoeksema et al., 2010; Kiers et al., 2011; Wagg et al., 2011; Fellbaum et al., 2014).

Another important group of fungi that can be host-specific are soilborne root pathogens, which also colonise roots and cause disease (Agrios, 2005; Sieber and Grünig, 2006; Doehlemann et al., 2014). Soilborne pathogens are often facultative saprophytes that can survive on dead organic matter if an appropriate host is not available (Jarosz and Davelos, 1995; Agrios, 2005). Others can survive dormant in the soil as spores or sclerotia for many years (Agrios, 2005). The accumulation of host-specific pathogens is a key reason behind crop rotations in agricultural fields, but generalist soilborne pathogens can also cause mortality and alter plant community structure and composition (van der Putten et al., 1993; Jarosz and Davelos, 1995; Mills and Bever, 1998; Mordecai, 2011).

Many plant-soil feedback studies have shown that invasive plants can alter soil microbial communities and generally experience positive feedback in their invasive range, particularly in regard to AM fungi (Mummey and Rillig, 2006; Reinhart and Callaway, 2006; Pringle et al., 2009; Zhang et al., 2010; van der Putten et al., 2013). However, there are increasing observations of pathogen accumulation on invasive plants, and, if this occurs in soils, it may lead to negative feedback and possibly even declines in invasive plant abundance and spread (van der Putten et al., 1993; Simberloff and Gibbons, 2004; Diez et al., 2010; Dostál et al., 2013; Flory and Clay, 2013). Invasive plants could also associate with fungi that are more pathogenic to their co-occurring native plants than to themselves (Eppinga et al., 2006). If these pathogens “spillover” to the native plants, then this could lead to apparent competition from which the invader benefits (Daszak et al., 2000; Mangla et al., 2008; Beckstead et al., 2010; Li et al., 2014). The temporal scale required for plant-soil feedbacks to develop after plant invasion and affect co-occurring native plants is unclear (Kardol et al., 2013). In addition, knowledge of concurrent changes in microbial communities during this process is lacking.

It is clear that fungi that associate with plant roots can range from mutualistic to pathogenic, and may also fall anywhere along a continuum between these functions. In this thesis I use the all-encompassing term “root-associated fungi”, because “endophyte” often refers to fungi that have no effect on plant growth and does not include mycorrhizas (Brundrett,

2004; Schulz and Boyle, 2006; Porras-Alfaro and Bayman, 2011). Root-associated fungi include all taxa that colonise the root and may or may not be mutualistic or pathogenic, encompassing mycorrhizas, pathogens, saprophytes, commensalists, and fungi of unknown function. The increasing availability and affordability of next generation sequencing platforms has made identifying these organisms much easier, enabling us to open the black box of root-associated fungi (Öpik et al., 2009; Blaalid et al., 2012; Lindahl et al., 2013; Nilsson et al., 2014).

Vincetoxicum rossicum (Kleopow) Barbar. (Apocynaceae) (syn. *Cynanchum rossicum* (Kleopow) Borhidi; dog-strangling vine) is a highly invasive plant in parts of North America, including southern Ontario in Canada and New York State in the USA (Sheeley and Raynal, 1996; Cappuccino et al., 2002). It associates with many root-associated fungi in its invaded range and has high rates of colonisation by AM fungi (Smith et al., 2008; Bongard et al., 2013). *Vincetoxicum rossicum* is originally from the Kharkov region in the Ukraine, and also occurs in southwest Russia (Pobedimova 1952 in DiTommaso et al., 2005a). The first record of *Vincetoxicum* in Ontario was by Mrs. A. G. White in 1889 at Toronto Junction (Moore, 1959). It was subsequently reported to be abundant in the Don Valley in Toronto in the early 20th century (Scott, 1913). By the 1970s it was widespread through southern Ontario and its 'weediness and potential nuisance value' was noted (Pringle, 1973: 27). *Vincetoxicum rossicum* is now a primary target for invasive species management in this region (Miller et al., 2007; Anderson, 2012). In North America, *V. rossicum* can establish under a range of light, moisture and climate conditions, as well as in many different vegetation types (DiTommaso et al., 2005a; Miller et al., 2007; Averill et al., 2010; Kricsfalusy and Miller, 2010; Anderson, 2012; Sanderson and Antunes, 2013). St Denis and Cappuccino (2004) proposed that ants were the main pollinators, but the plant is highly self-compatible. They found no significant differences in seed set between self-pollinated, cross-pollinated and unmanipulated control plants. Germination rates range between 30 and 50% and one stem is capable of producing up to 400 viable seeds per year, which can be polyembryonic (Cappuccino et al., 2002; DiTommaso et al., 2005b; Douglass et al., 2009; Averill et al., 2011).

1.1. Aim and objectives

This thesis is arranged as three main chapters in manuscript-style ready for peer reviewed journal submission; specific theory and aims for each chapter are stated therein. The overall aim of this thesis was to investigate temporal dynamics and the functional ecology of root-associated fungal communities on the invasive plant, *Vincetoxicum rossicum* (Apocynaceae), in southern Ontario. I used a combination of field, glasshouse, and laboratory experiments to fulfill the following objectives:

1) To investigate if *V. rossicum* established associations with a specific group of root-associated fungal taxa (chapters 2 and 3);

2) To investigate temporal changes in richness and composition of root-associated fungal communities over the course of invasion, ranging from fine scale changes of weeks to longer term changes over decades after initial invasion using an invasion chronosequence approach (chapters 2 and 3);

3) To test whether pathogen accumulation and stronger negative feedback occurred as invasion progressed over decades using an invasion chronosequence (chapter 3);

4) To investigate effects of fungi isolated from roots of *V. rossicum* on the growth of their host and co-occurring native plants, *Asclepias syriaca* and *Solidago canadensis* (chapter 4).

In chapter 2 I investigated fine scale temporal patterns in AM fungal communities in *V. rossicum* using soil collected from previously invaded and uninvaded locations at two sites. Plants were grown in the glasshouse for 29 weeks and AM fungal composition was assessed every 4-6 weeks using Denaturing Gradient Gel Electrophoresis (DGGE). Investigating patterns over longer time scales in chapter 3, I combined a plant-soil feedback study with high-throughput sequencing (454 pyrosequencing) to investigate changes in plant growth and root-associated communities. A space-for-time substitution approach was used, where plants and soil were collected from 20 sites representing *V. rossicum* invasion from 0 to approximately 100 years. In Chapter 4, I isolated and identified root-associated fungi from *V. rossicum*. Seven fungal isolates that are known pathogens on other plant species were tested for growth effects

on *V. rossicum*, and three were tested on two commonly co-occurring native plant species, *Asclepias syriaca* (Apocynaceae) and *Solidago canadensis* (Asteraceae).

Chapter 2. Temporal effects of an invasive plant on arbuscular mycorrhizal fungal communities

2.1. Abstract

The composition of arbuscular mycorrhizal (AM) fungal communities can be altered by invasive plants, however, little is known about the timescale of these changes. Using a glasshouse study with soil from two sites, I investigated fine-scale temporal changes in AM fungi colonising the exotic invasive plant *Vincetoxicum rossicum* (Apocynaceae), using molecular techniques targeting the large subunit of ribosomal DNA. I hypothesised that AM fungal communities in the roots of *V. rossicum* growing in previously invaded and uninvaded soils would differ initially and there would be positive feedback. However, I expected AM fungal composition of plants in the uninvaded soil to rapidly converge towards that of plants in the invaded soil. *Vincetoxicum rossicum* was colonised by many AM fungal isolates quickly and most of these remained in the plant throughout the 29 weeks of the study. Initially, AM fungal composition in plants differed significantly between soil treatments. Plants in invaded soil were more similar in AM fungal composition than those in uninvaded soil, but there was no convergence in AM fungal composition in plants in uninvaded and invaded treatments over this 29 week study. These results indicate that AM fungal communities may change in the presence of an invasive plant, but this process requires a longer period of time than the first growing season after establishment.

Key words: plant invasion; arbuscular mycorrhizal (AM) fungi; temporal; *Vincetoxicum rossicum*; dog-strangling vine; denaturing gradient gel electrophoresis.

2.2. Introduction

Arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) establish obligate mutualisms with most land plants, in which plants provide the AM fungi with carbon in exchange for benefits including nutrient uptake and pathogen protection (Smith and Read, 2008). The symbiosis falls along a continuum from mutualism to parasitism (Johnson et al., 1997; Klironomos, 2003; Kiers et al., 2011); however, a meta-analysis indicated that AM fungal associations are generally beneficial in terms of increasing plant biomass (Hoeksema et al., 2010). AM plants are colonised by multiple fungal species and species isolates, with some more beneficial for plant growth and other responses than others (van der Heijden et al., 1998; Eom et al., 2000; Lewandowski et al., 2013). Feedback and selective recruitment between particular plant species and AM fungal isolates has been demonstrated (Pringle et al., 2009; Kiers et al., 2011; Fellbaum et al., 2014), which may lead to positive feedback in terms of plants having higher biomass in soil where they have previously grown (Bever et al., 2012). AM fungal community structure is determined by a range of biotic and abiotic factors, including plant host (van der Heijden et al., 1998; Eom et al., 2000; Lekberg et al., 2007; Jansa et al., 2008; Hausmann and Hawkes, 2010), its neighbours (Hausmann and Hawkes, 2009; Lekberg et al., 2012, 2013), land use, and soil type and pH (Lekberg et al., 2007; Schreiner and Mihara, 2009; Oehl et al., 2010; Bunn et al., 2014).

Studies using molecular tools have also shown seasonal changes to be important drivers of changes in AM fungal communities (Liu et al., 2009; Dumbrell et al., 2011; Sánchez-Castro et al., 2012; Helgason et al., 2014), while others have shown no seasonal relationships (Rosendahl and Stukenbrock, 2004; Santos-González et al., 2007). Plant growth strategies, may play a role; within one growing season, Daniell et al. (2001) found that AM fungal diversity was highest in the middle of the growing season in four annual crop species, but Helgason et al. (2014) found that species richness in the woody perennial *Acer* increased throughout the growing season. How long it takes for AM fungal richness or composition to change over time is unknown, but Zhang et al. (2010) showed that AM fungal composition was altered over two growing seasons in the presence of the herbaceous perennial *Solidago canadensis*. Fine scale temporal changes in AM fungal communities associating with invasive plants in their introduced range have not been investigated. Since AM fungi can be associated with enhanced plant

growth and fitness, understanding the time required for plant-soil feedbacks to develop may help to predict the spread and long term impacts of plant invasions (Levine et al., 2006).

A primary goal in conservation is to reduce the abundance of invasive plants due to their capacity to form dense stands and negatively impact native communities (Petitpierre et al., 2012; Simberloff et al., 2013). What causes only some exotic plants to become invasive is complex and continues to be investigated (Pyšek et al., 2009; Simberloff et al., 2013; Kuester et al., 2014). Invasive plants often demonstrate positive feedback with soil biota in their invaded range (e.g., van der Putten et al., 2007a; Pendergast et al., 2013). Invasive plants can be dependent on mutualisms in their native range, but this may not hinder invasion success in a novel range (Richardson et al., 2000; Moora et al., 2011; Wandrag et al., 2013; Nuñez and Dickie, 2014). AM fungi are increasingly considered to be important determinants of plant community structure and there is evidence that they contribute to the success of invasive plants (Halvorson and Koske, 1987; Klironomos, 2002; Pringle et al., 2009; Moora et al., 2011; Nuñez and Dickie, 2014). One theory is that successful invasive plants that are highly mycorrhizal (i.e., obtain a large fitness benefit from associating with mycorrhizas) should be able to associate with a wide range of AM fungal species so that the ability to form mutualisms is not a limitation to range expansion (van der Putten et al., 2007b; Pringle et al., 2009; Nuñez and Dickie, 2014). Zhang et al. (2010) demonstrated positive feedback in an invasive plant in China, where an AM fungal species that increased biomass of *Solidago canadensis* was in higher abundance in this plant species compared to a neighbouring native plant species. However, little is known about how mycorrhizal invasive plants alter AM fungal communities over time and how quickly these changes may occur (Kardol et al., 2013).

Plant host identity is an important determinant of AM fungal community composition (Eom et al., 2000; Santos-González et al., 2007; Jansa et al., 2008; Hausmann and Hawkes, 2010). An understanding of the changes in AM fungal communities in invasive plants immediately after introduction and as they expand may be useful for understanding whether this is an important mechanism of invasion (Hawkes et al., 2006; Mummey and Rillig, 2006; Zhang et al., 2010; Barto et al., 2011; Nuñez and Dickie, 2014). Understanding how long it may take for invasive species to alter indigenous AM fungal communities is important from both ecological and management perspectives. For instance, eradication response times and potential soil restoration methods could be enhanced by knowledge of the impacts of invasive plants on

belowground biodiversity. If invasive plants are highly responsive to the AM fungal symbiosis and recruit particular AM fungal species then it is expected that the AM fungal composition of plants growing in uninvaded soil would rapidly converge towards that of plants growing in previously invaded soil. These rapid changes in soil microbial communities are the basis of many plant-soil feedback studies where soil is trained (Callaway et al., 2004; van der Putten et al., 2007a; MacDougall et al., 2011).

Vincetoxicum rossicum (Kleopow) Barbar. (Apocynaceae) (syn. *Cynanchum rossicum* (Kleopow) Borhidi; dog-strangling vine) is a highly invasive plant in parts of North America, including southern Ontario in Canada (Sheeley and Raynal, 1996; Cappuccino et al., 2002). It becomes dominant and can outcompete surrounding vegetation (Cappuccino, 2004; Douglass et al., 2009; Anderson, 2012). Originally from the Ukraine and southwest Russia (Pobedimova 1952 in DiTommaso et al., 2005a), *V. rossicum* in North America can establish under a range of light, moisture, and climate conditions, as well as in many different soil and vegetation types (DiTommaso et al., 2005a; Averill et al., 2011; Sanderson and Antunes, 2013). *Vincetoxicum rossicum* is highly mycorrhizal dependent, showing significantly higher biomass when AM fungi are present compared to absent (Smith et al., 2008). In addition, *V. rossicum* readily associates with many AM fungal species in its invaded range (Bongard et al., 2013). These characteristics indicate that *V. rossicum* may demonstrate positive feedback.

The objective of this study was to determine whether invasion by *V. rossicum* rapidly alters AM fungal community composition (within one growing season). I collected soil from two sites with areas invaded by *V. rossicum* and adjacent uninvaded areas with the same soil type. *V. rossicum* was grown in these soils in a glasshouse and changes in the AM fungal communities colonising roots were regularly assessed over 29 weeks. The hypothesis was that AM fungal species colonising plants in invaded and uninvaded soils are different, and that plants in the invaded soil would have higher biomass due to positive feedback, based on previous studies (Mummey and Rillig, 2006; Zhang et al., 2010; Pendergast et al., 2013). However, over time, if *V. rossicum* alone is a strong driver of AM fungal community composition then I expected community composition in plants growing in uninvaded soil to converge towards that in plants from invaded soil due to selective recruitment by *V. rossicum* (Mummey and Rillig, 2006; Zhang et al., 2010; Bever et al., 2009; Kiers et al., 2011).

2.3. Methods

2.3.1. Soil and seed collection

Soil was collected from the Toronto Zoo, Ontario, Canada (N 43°49'7", W -79°11'8") at each of two sites, which were approximately 1 km apart. Within sites, soil was collected from two paired locations: one with no record of *V. rossicum* invasion ('uninvaded' treatment) and the other with a dense population of *V. rossicum* ('invaded' treatment). *Vincetoxicum rossicum* had been present for at least 20 years in the invaded locations, which were last mown or managed in the early 1990s (J. Bell, Toronto Zoo, personal communication). The two sites were chosen within a small geographic area to minimise environmental and soil differences, and ensure representative adjacent uninvaded areas with similar management histories were available. Plant species composition was assessed at each location by establishing a 16 m^2 plot and dividing it into 16 1 m^2 quadrats. Presence of each plant species within each quadrat was recorded. It is assumed the plant communities in the uninvaded locations are representative of those present prior to invasion by *V. rossicum* and that all locations were at the same successional stage, and should also be noted that the uninvaded locations were not dominated by natives. In each location, approximately 60 L soil was collected with a spade from the top 20-30 cm, covering an area of approximately 3 m^2. Soil was homogenised by sieving (4 mm) and placed in air tight, opaque containers for transport back to the laboratory for storage at 4ºC until the start of the experiment four days later. At the sites of collection, all containers, spades and soil sieves were scrubbed and soaked in diluted bleach for at least 20 minutes to prevent cross-contamination between locations. Rubber gloves were changed between treatments and thoroughly sprayed with 70% ethanol. A subsample of soil from each location indicated that both sites had the same soil type: a Till Plain slightly alkaline (pH 8) fine sandy loam. Soil fertility was similar among all four locations (Table S 2.1).

Seeds of *V. rossicum* were collected five weeks prior to soil collection from opened seedpods within the invaded locations at both sites. Pappi were removed and seeds were placed in paper envelopes for storage at 4ºC for approximately two weeks. Seeds from one plant at each site were placed between sheets of moist filter paper and stratified in the dark at 4ºC for 18 days (Smith et al., 2008). Prior to planting, seeds were surface disinfected in 10% bleach for three minutes followed by rinses in sterile water.

2.3.2. Experimental design

The experimental design was a completely randomised factorial experiment with two crossed factors: treatment (soil with two levels: invaded and uninvaded) and time (with 5 levels: harvests 1, 2, 3, 4 and 5, corresponding to 9, 13, 19, 24, and 29 weeks after planting) with four replicates per treatment-time combination. I also prepared four sterile control pots for each treatment, consisting of autoclaved soil (90 minutes at 121ºC and 18 psi), making a total of 16 controls and 96 experimental units overall. To minimise cross contamination, all potting equipment was disinfected by soaking in diluted bleach for at least 20 minutes, benches were scrubbed with bleach and rinsed, and rubber gloves were changed between potting locations. For each location, soil was sieved and mixed with sterile sand (non-calcareous “B” sand, Hutcheson Sand and Mixes, Huntsville, ON, Canada) and turface (calcined, non-swelling illite and silica clay, Turface Athletics MVP, Profile Products LLC, Buffalo Grove, IL, USA) in a 1:1:1 ratio. This substrate was divided equally into 2.8 L pots (Nursery products Inc., C300 pots 18 cm tall 16 cm diameter). Control pots contained sterile soil, sand, and turface in a 1:1:1 ratio and were used to assess potential cross contamination or glasshouse effects. Pots were lined with 2 mm mesh to prevent substrate loss and placed on saucers.

Four *V. rossicum* seeds were placed into each pot approximately 5 mm below the soil surface using sterile tweezers. Where multiple seeds germinated in a pot, one seedling was randomly selected to grow and the other seedlings were repeatedly cut at soil level using sterile scissors. Plants other than *V. rossicum* were pulled out immediately after germinating. Pots were randomised monthly to account for possible spatial effects in the glasshouse. Plants were watered with reverse osmosis water between two and seven days each week over the course of the experiment and received a 14/10 day/night photoperiod with temperature ranging between 20 and 24ºC. At week 11 (between harvests 1 and 2) 200 ml of low phosphorus fertiliser was added to each pot as they were showing signs of nutrient deficiency (20-2-20 NPK, N 20 ppm; Plant Products, Brampton, Ontario, Canada).

2.3.3. Harvest

Four randomly selected replicates were harvested from each location 9, 13, 19, 24, and 29 weeks after planting (harvest times 1, 2, 3, 4, and 5). The thorough homogenisation of soil when potting means that these pots were likely to be representative of all pots from each location. All controls were harvested at harvest 5. Prior to the first harvest, I verified that AM fungi had colonised by clearing and staining roots of four plants randomly selected from each site and treatment combination. Equipment was disinfected between each replicate using the same methods as when potting. Harvested plants were stored at 4°C for no longer than two days prior to processing. A sterile scalpel was used to separate roots and shoots. Roots were thoroughly washed free of soil in tap water, patted dry with a clean paper towel, and weighed. A subsample of approximately 100 mg of roots was selected at random, cut into 1-2 cm pieces, placed in sterile 2 ml microcentrifuge tubes, and frozen at -80°C for subsequent DNA extraction. Where possible, two 100 mg root samples were taken at random from each plant to capture as much of the AM fungal community as possible and to account for potential biases during DNA extractions (74/80 samples). Shoot and root biomass were separated and dried at 60°C for at least three days and weighed. Because root subsamples were taken for DNA extraction, total dry root biomass was estimated as follows: Dry weight = dry mass + ((fresh mass – fresh mass after subsample taken) / fresh mass) * dry mass). All plants were weighed twice to ensure consistency in dry weights.

2.3.4. DNA extraction from roots

Frozen roots were homogenised by placing four sterilised 3 mm tungsten carbide beads into each microcentrifuge tube with the roots, immersing in liquid nitrogen for 30 seconds and shaking at 30 mHz for one minute in a MoBio 96 Well Plate Shaker (MoBio Laboratories, Carlsbad, CA, USA). These steps were repeated a total of six times to ensure sufficient root homogenisation. Roots did not thaw during this process. Subsequent DNA extraction was done using the Macherey-Nagel Nuceleospin Mini kit using CTAB cell lysis buffer (Macherey-Nagel and Co. KG, Düren, Germany). Beads were sterilised between extractions by washing with deionised water, soaking in an agent to remove nucleases and nucleic acids (UltraClean Lab Cleaner, MoBio Laboratories, Carlsbad, CA, USA), rinsing thoroughly and autoclaving at 121ºC

and 18 psi for 15 minutes. Where two root samples were from the same individual, DNA was pooled. DNA was stored at -20°C.

2.3.5. Polymerase Chain Reaction

Plant DNA was amplified by nested polymerase chain reaction (PCR) using primers targeting the large ribosomal subunit (LSU) genes. Primer set LR1 (GCATATCAATAAGCGGAGGA; van Tuinen et al., 1998) and FLR2 (GTCGTTTAAAGCCATTACGTC; Trouvelot et al., 1999) were used to first amplify all fungi. The PCR mix was composed of 500 nM each primer, 4 µl 5X buffer 25 mM magnesium chloride 200 nM dinucletotide triphosphate mix 1.25 units of GoTaq Flexi polymerase enzyme (Promega, Madison, WI, USA) and 1 µl template DNA from roots. Total volume was made up to 20 µl with sterile DNase-free water. The PCR program was an initial cycle of DNA denaturation at 95ºC for 3 minutes, 57ºC for 30 seconds and elongation at 72 ºC for 1 minute, followed by 30 cycles of denaturation at 95ºC for 1 minute, annealing at 57ºC for 30 seconds and elongation at 72 ºC for 1 minute. The last cycle was followed by a 5 minute elongation at 72 ºC. The PCR product was diluted 1:999 with sterile water and used in subsequent reactions with the same PCR protocol and primers FLR3 (TTGAAAGGGAAACGATTGAAGT; Gollotte et al., 2004) and FLR4 (TACGTCAACATCCTTAACGAA; Gollotte et al., 2004) to target AM fungi. This dilution was used because 1:9 and 1:99 dilutions resulted in smeared or multiple bands, probably due to excessive DNA. A GC clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG; Muyzer et al., 1993) was added at the 3' end of the FLR3 primer to prevent complete denaturation during subsequent denaturing gradient gel electrophoresis (DGGE). FLR3 and FLR4 are known to preferentially amplify some groups in the phylum but are still widely used (Mummey and Rillig, 2007; Krüger et al., 2012). Sizes and quality of PCR products were assessed by running on 1% agarose gels and viewing under UV light. Each PCR run had at least one positive AM fungal control (DNA extracted from spores of *Rhizophagus irregularis* DAOM 197198) and one negative control (sterile DNase-free water) used in place of the template DNA. Two 20 µl PCR reactions were run for each sample and products were pooled for DGGE to mitigate PCR bias and increase the

probability of capturing the entire AM fungal community. There was no amplification of fungal or AM fungal DNA in the roots from the control pots.

2.3.6. Denaturing Gradient Gel Electrophoresis

Four DGGE gels were run for band analysis, one for each location, with four replicates of each of the five harvest times for a total of 80 samples. A D-Code system was used to perform the DGGEs (BioRad, Hercules, CA, USA). Gels contained 8% acrylamide with a gradient of 20-50% denaturant. PCR products were mixed with 8 µl of loading dye and a total volume of 35 µl PCR product and dye mix was loaded into each well. Gels were run at 20 mV until the temperature reached 65°C (approximately 10 minutes) then voltage was increased to 75 mV and run for 17 hours. Gels were stained with 0.02 SYBR green for 15 minutes, UV-illuminated and photographed using GeneSnap (Syngene, Cambridge, UK). Prominent bands were excised from the gel, placed in 20 µl sterile water, and amplified using the same PCR protocol as above using the FLR3 and FLR4 primers without the GC clamp. PCR products were purified (GenElute PCR Clean-Up Kit, Sigma-Aldrich, Saint Louis, MO, USA) and sequenced by Sanger sequencing (Sanger et al., 1977) at the Genomics Facility in the Advanced Analysis Centre at the University of Guelph (http://www.uoguelph.ca/~genomics/Genomics%20Facility.htm). Sequences have been submitted to Genbank (Accession Numbers KM391837- KM391861).

2.3.7. Gel analysis

AM fungal data for richness and composition were obtained from pictures of the four DGGE gels and imported into GeneTools (Syngene, Cambridge, UK). Gels were aligned using internal standards based on multiple preliminary gels. The presence and absence of each band in each plant sample (gel lane) was determined. AM fungal band richness was calculated by summing the number of bands in each plant sample. I used presence/absence of bands and did not use DGGE band fluorescence intensity as a measure of abundance, due to inconsistencies in band fluorescence between PCR products (Nakatsu, 2007).

2.3.8. Phylogenetic analysis of excised DGGE bands

DNA sequences were aligned and a phylogenetic tree was drawn using MEGA version 5.2 (Tamura et al., 2011) following methods described by Hall (2000). Sequences from the excised DGGE bands were blasted against Genbank using the default settings and the FASTA files of the most closely related published sequences were downloaded. In addition, known sequences within the Glomeromycota were downloaded for inclusion in the phylogenetic tree. All sequences were aligned and trimmed to keep only the LSU portion. Primer and duplicated sequences were deleted. DGGE-excised sequences were aligned and drawn into a neighbour-joining tree constructed under the Maximum Composite Likelihood model, which is a likelihood version of the Tamura-Nei model (Hall, 2000). This model accounts for differences between the different types of substitutions in purines and pyrimidines. That is, it is more common for a transition mutation to occur because the shape of the DNA strand will be maintained (purine substituted for purine or pyrimidine for pyrimidine; Hall, 2000). Tree reliability was assessed by 2000 bootstrap replications and *Scutellospora* sp. was used as outgroup to root the tree (Hall, 2000) because it belongs to a different Order than all the other sequences obtained.

2.3.9. Data analysis

All statistical analyses were performed in R version 3.0.2 (R Core Development Team, 2013) using packages where specified. All bands, sequenced and unsequenced (n=44), were used to investigate AM fungal community structure. AM fungal band composition was visualised for all sites, treatments, and harvests by using principal co-ordinates analysis (PCoA) with 6 dimensions using function ‘cmdscale’ in base R. Jaccard’s distance was used because it is appropriate for presence/absence data and absence of a band does not contribute towards similarity of samples (McCune and Grace, 2002; Legendre and Legendre, 2012). The advantage of PCoA is that there is no underlying assumption of linearity between species distributions and the environment (McCune and Grace, 2002). If *V. rossicum* had been colonised by a particular composition of AM fungi, I expected there to be less variability in

composition in the invaded treatment than the uninvaded treatment across sites. I tested this by using a multivariate test of homogenous variances (Anderson, 2006). This was done using the 'betadisper' function with Jaccard's distance, and significance of the *F*-statistic was tested with 'permutest' and 999 permutations under the null hypothesis of no difference in dispersion between treatments in vegan package version 2.0-7 (Oksanen et al., 2013a). The multivariate test of homogeneous variances has no assumptions of a multivariate distribution. Its only assumpation is that points are exchangeable in multivariate space under the null hypothesis of equal dispersion (Anderson, 2006).

Given the site differences in plant composition (Table S 2.2), I separated AM fungal band data by site to investigate patterns in AM fungal richness and composition. To investigate differences in AM fungal richness over time and between treatments, band richness was used as the response variable in generalised linear models for each site using function 'glm' specifying the Poisson link for count data (Crawley, 2002), including an interaction term between treatment and harvest. Each model met the assumptions of independence and the variance was equal to the mean. To investigate the significance of treatment and time on band composition, a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) specifying Jaccard's distance was performed for each site, including an interaction term between treatment and harvest. PERMANOVA is a robust method to partition variance between groups in multivariate data and produces pseudo *F*-statistics and *P*-values based on multiple random permutations of the given dissimilarity matrix (Anderson, 2001). *P*-values were estimated from 1000 randomisations using the 'adonis' function in vegan package version 2.0-7 (Oksanen et al., 2013a). The only assumption for PERMANOVA is that observations are independent (Anderson, 2001).

To investigate plant growth responses, two generalised linear models were run for each site with different response variables: total biomass and root-shoot ratio. The Gaussian link was specified for both for continuous data and the assumptions of independence and homoscedasticity was met for both models (Crawley, 2002). Models were performed using function 'glm', specifying an interaction between treatment and time. Only data for 60 plants at harvests 2, 3, 4 and 5 were included in this model because all the root biomass from samples from harvest 1 and four plants from harvest 2 were used for DNA extractions (two from each treatment).

2.4. Results

2.4.1. Did AM fungal composition differ between invaded and uninvaded treatments?

A total of 44 unique bands were distinguished across all 80 samples. There were between 7 and 18 AM fungal bands per plant, with a mean of 12 ± 0.3 bands per plant, and there was no significant difference in AM fungal band richness between treatments or harvests at either site (results not shown). The PCoA explained 51.8% of the variation in AM fungal composition and showed that AM fungal composition was different between sites and treatments (Fig. 2.1). Most bands were present in both sites, indicating that the differences between sites in the PCoA were driven by rare bands. Of the 44 distinct bands across samples, seven were unique to site 1 and six to site 2. Five bands occurred only in the invaded treatment, while nine bands were unique to the uninvaded treatment across all sites.

Separating by site, the PERMANOVAs showed that treatment explained a significant amount of variation in AM fungal composition (Table 2.1). At site 1, AM fungal composition changed significantly over time, and there was a significant interaction between time and treatment; AM fungal composition diverged between treatments at harvest 5. The treatment by time interaction was marginally significant for site 2 (P=0.059); AM fungal composition diverged between treatments at harvests 2 and 5 (Table 2.1). All of the 25 sequenced bands corresponded to AM fungi in the Order Glomerales of Phylum Glomeromycota (Fig. 2.2, Table S 2.3).

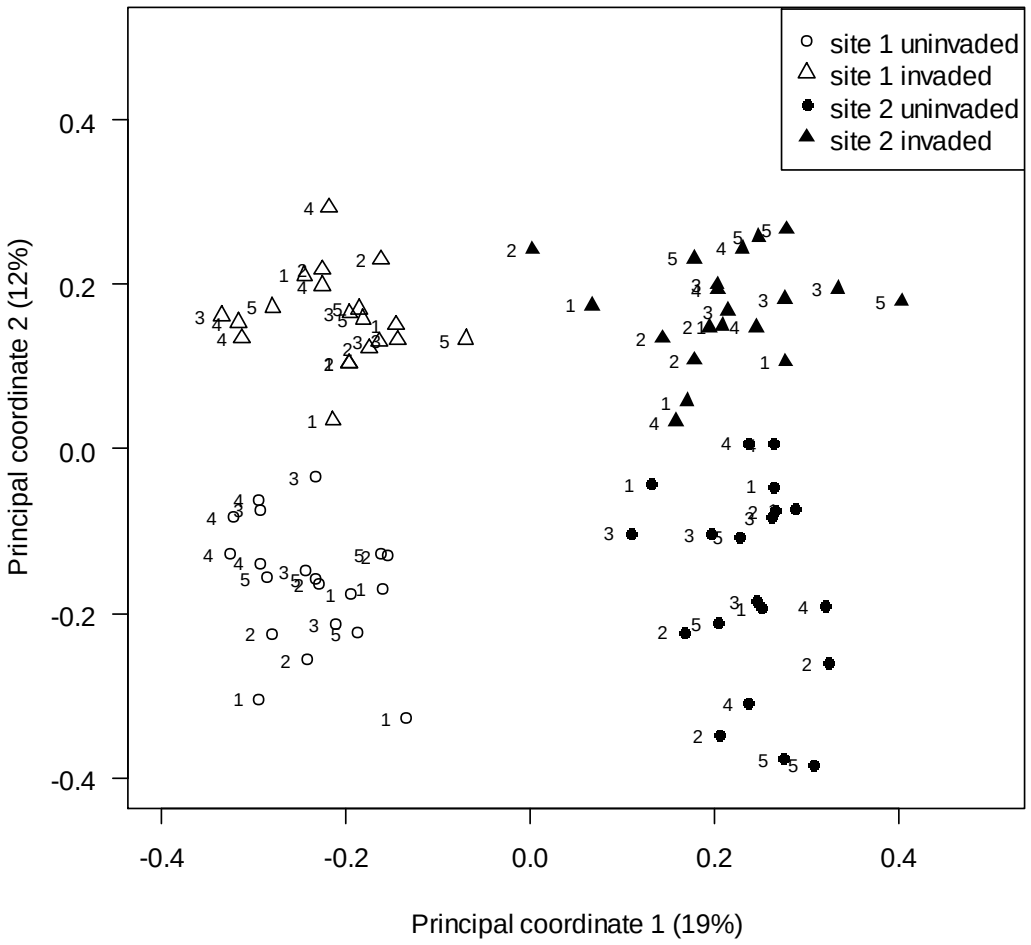

Fig. 2.1 Site scores of principal co-ordinates analysis (PCoA) using Jaccard's distance on AM fungal bands (n=44) from denaturing gradient gel electrophoresis (DGGE), showing sites and treatments across all harvest times. Values in brackets on axes correspond to the amount of variation explained by each axis. Numbers next to points represent harvest number.

Table 2.1 Results from permutational analysis of variance (PERMANOVA) for each site with Jaccard's distance on AM fungal composition based on DGGEs.

Variable	Variation explained (%)	df	SS	MS	Pseudo *F*	*P*
Site 1						
Treatment	23	1	1.71	1.71	13.19	0.001
Time	12	4	0.94	0.24	1.82	0.004
Treatment × time	14	4	1.08	0.27	2.08	0.001
Residuals	51	30	3.88	0.13		
Total	100	39	7.60			
Site 2						
Treatment	25	1	1.73	1.73	13.22	0.001
Time	8	4	0.56	0.14	1.07	0.372
Treatment × time	11	4	0.76	0.19	1.45	0.059
Residuals	56	30	3.93	0.13		
Total	100	39	6.98			

2.4.2. Did AM fungal community composition between invaded and uninvaded soil converge over time?

Across both sites, there was a significant change in AM fungal composition over time but no convergence in composition between invasion treatments (Table 2.1, Fig. 2.1). The multivariate test for homogeneity of variances between treatments showed that overall, AM fungal composition in plants in the invaded treatment were more similar (i.e., there was less variation) than those in the uninvaded treatment ($F_{1,38}$ = 17.74, P<0.001, 999 permutations; Fig. 2.1).

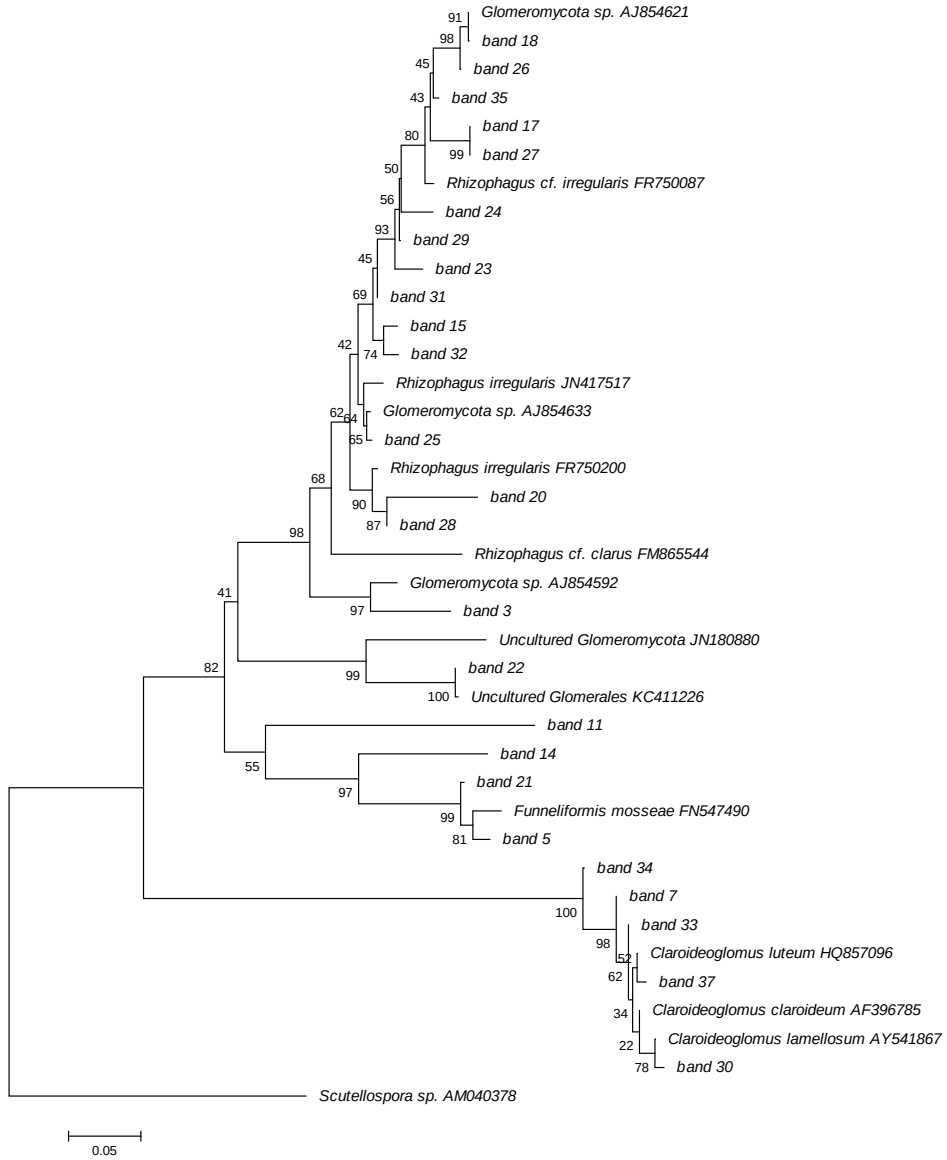

Fig. 2.2 Neighbour-Joining phylogenetic tree showing evolutionary relationships between sequenced DGGE bands and Genbank sequences. The optimal tree with the sum of branch length = 2.017 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 iterations) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 39 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 340 positions in the final dataset. All sequences obtained were in the Order Glomerales. *Scutellospora* sp. in the Diversisporales was used to root the tree.

2.4.3. Were there differences in plant biomass between treatments?

Plant growth differed depending on invasion treatment. Plants in the invaded treatment had consistently higher total biomass than those in the uninvaded treatment, but this was only statistically significant for Site 2 after harvest 2 (Table 2.2, Fig. 2.3). At both sites, plants in the uninvaded treatment had higher root-shoot ratios than those in the invaded treatment. There was also a significant interaction between treatment and time for root-shoot ratio at both sites (Table 2.2); while the root-shoot ratio of plants growing in the invaded treatment remained relatively constant, the allocation of biomass to root relative to shoot growth was greater in plants in the uninvaded treatment after harvest 3 (Table 2.2, Fig. 2.3).

Table 2.2 Results from generalised linear model for total biomass and root-shoot ratio from harvests 2 to 5 for each site.

Predictor	Total biomass			Root-shoot ratio		
Site 1	Coefficient	*t*	*P*	Coefficient	*t*	*P*
Intercept	-2.95 (1.06)	-2.80	0.009	-0.48 (0.47)	-1.01	0.321
Treatment	0.45 (1.43)	0.32	0.755	-1.32 (0.64)	-2.07	0.048
Time	0.30 (0.05)	6.32	<0.001	0.10 (0.02)	4.55	<0.001
Treatment × time	-0.05 (0.06)	-0.78	0.440	0.09 (0.03)	3.14	0.004
Site 2						
Intercept	-4.72 (0.56)	-8.43	<0.001	0.25 (0.65)	0.38	0.706
Treatment	1.64 (0.84)	7.94	0.063	-2.73 (0.98)	-2.79	0.010
Time	0.41 (0.02)	16.64	<0.001	0.07 (0.03)	2.26	0.032
Treatment × time	-0.16 (0.04)	-4.28	<0.001	0.16 (0.04)	3.73	0.001

Note: Maximum likelihood coefficients and standard errors (±) are presented.

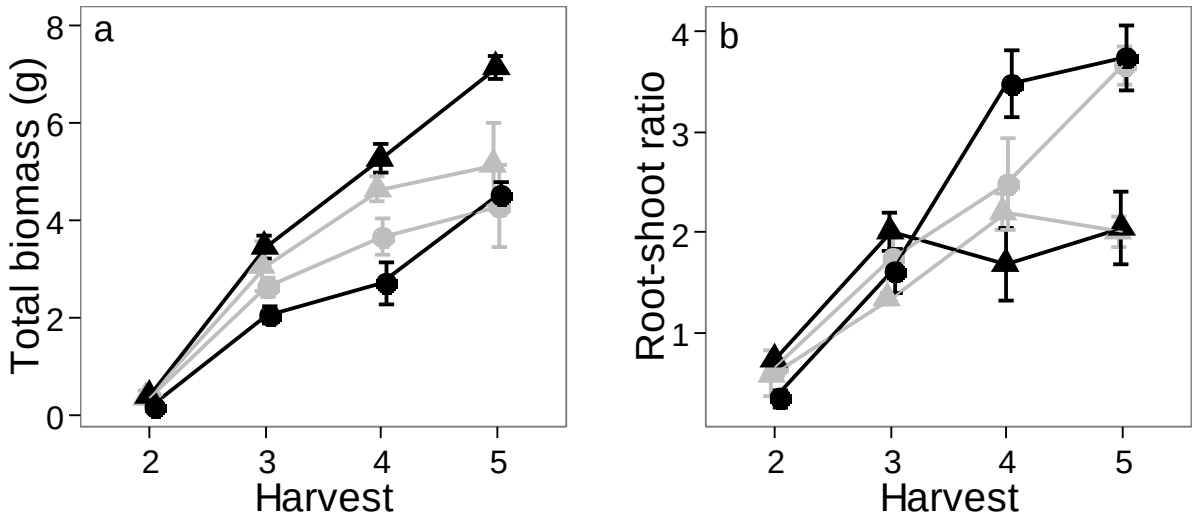

Fig. 2.3 (a) Total biomass (g) and (b) root-shoot ratios from harvests 2 to 5 for *Vincetoxicum rossicum* plants by site and treatment. Grey lines represent plants in Site 1, black lines represent plants in Site 2, circles represent the uninvaded treatment, triangles represent the invaded treatment. Values are means ± standard errors. Graphs were produced in R using package 'ggplot2' (Wickham 2009).

2.5. Discussion

AM fungal composition differed where *V. rossicum* had invaded for multiple decades compared to where it had not at the two sites represented here. However, such differences were not apparent in the relatively short-time frame of the first growing season after establishment because convergence in AM fungal community composition between treatments was not detected over the 29 weeks of this study across both sites. This indicates that either AM fungal communities need longer than one growing season to change due to the presence of *V. rossicum* or that factors other than, or in addition to, host species drive these changes. However, the overall similarity in trends at these two sites suggests that *V. rossicum* is important for AM fungal community composition. Although plant species composition differed between sites and locations (Table S 2.1), the homogeneity of variances test showed that AM fungal species composition in the invaded treatment was more similar than that of the uninvaded treatment across both sites. In addition, the PERMANOVAs showed that invasion

treatment accounted for a substantial proportion of variation in AM fungal composition: 23% and 25% in Sites 1 and 2 respectively (Table 2.1). Due to differential growth effects of AM fungi on different plant species, these patterns may have consequences for future restoration of native plant communities after invasion for many years (Hawkes et al., 2006; Mummey and Rillig, 2006; Vogelsang and Bever, 2009).

AM fungal composition differed between site and invasion treatments (Table 2.1, Fig. 2.1). This could be a reflection of the differences in plant species composition between sampling locations (Table S 2.2). However, despite these differences, AM fungal composition and plant biomass responded similarly to *V. rossicum* within treatments for both sites in the glasshouse (Tables 2.1 and 2.2). Others have suggested that site history or soil texture and pH may be more important than plant host for determining AM fungal community composition (Lekberg et al., 2007; Sikes et al., 2012; Bunn et al., 2014). The site differences could be explained by spatial effects because spatial differences in AM fungal communities have been observed at fine scales of centimetres (Wolfe et al., 2007; Mummey and Rillig, 2008) and larger scales of metres (Rosendahl and Stukenbrock, 2004; Davison et al., 2012) and kilometres (Husband et al., 2002). Since plant composition differed between sites, the greater similarity of AM fungal composition in the invaded treatments across both sites suggests that *V. rossicum* may play a role in determining AM fungal communities over long time scales, or that these AM fungal communities are related to environmental factors. Mummey and Rillig (2006) also found that soil from areas invaded by *Centaurea maculosa* had similar AM fungal species richness, but differed in composition. Future work incorporating a greater number of sites across measured environmental gradients, including sites invaded by *V. rossicum* for different periods of time, will help to clarify the generality of the observed patterns.

There was some evidence for positive feedback; *V. rossicum* had higher biomass in the invaded treatment at Site 2 from harvest 2 through to the end of the experiment (Table 2.2, Fig. 2.3). This could be due to differences in AM fungal composition between treatments (Table 2.1), however, other biota were present in the soil and are likely to have contributed to the overall growth effect. In saying this, plants in the invaded treatment were more similar in AM fungal composition than plants in the uninvaded treatment, suggesting selective recruitment of particular AM fungal isolates which may contribute to the positive feedback response in Site 2. In addition, allometry differed by treatment; plants in uninvaded soil had a higher root-shoot ratio

than plants in invaded soil at both sites (Table 2.2, Fig. 2.3). These differences were unlikely to be caused by nutrients because fertiliser was applied to all pots two weeks prior to harvest 2 and the root-shoot ratios were still similar in the different treatments at harvest 2 (Fig. 2.3). Evidence is accumulating for bidirectional control of AM fungal colonisation of plants and species-specific preferences (Bever et al., 2009; Kiers et al., 2011; Fellbaum et al., 2014), which may explain the observed patterns and have wider ecological impacts in plant communities. For example, Zhang et al. (2010) showed that one AM fungal species present in high abundance in *Solidago canadensis* in its invasive range in China increased the host's competitive ability against the native *Kummerowia striata*.

I had hypothesised that AM fungal composition in the uninvaded treatment to converge towards that of the invaded treatment over the 29 weeks, which was based on previous studies showing differences in AM fungal communities in invaded and uninvaded areas (e.g., Mummey and Rillig, 2006). The lack of convergence in AM fungal composition between the uninvaded and invaded treatments across both sites over this 29-week study indicates that plant-induced changes in soil microbial communities may take multiple seasons to occur (Kardol et al., 2013; Hart et al., 2014) or that *V. rossicum* invasion is not the main driver of the observed patterns and that initial AM fungal composition is important for determining temporal trajectories in communities. *Artocarpus altilis* showed marked changes in AM fungal composition from seedlings to plants 32-40 years old (Hart et al., 2014), indicating that substantial changes in community composition may take many decades to occur. However, Zhang et al. (2010) found that AM fungal communities changed in response to *Solidago canadensis* in just two growing seasons. This glasshouse study under constant climatic conditions and homogenised substrate provided ideal conditions for observing convergence if it does occur over shorter time periods, because compositional convergence in AM fungal communities is most likely to occur under homogeneous environmental conditions (Caruso et al., 2012). These results have implications for plant-soil feedback studies that often train soil for shorter periods of time than the time-frame of this study. These results with *V. rossicum* indicate that 29 weeks may not be enough time for the AM fungal communities to converge towards those found colonising in older invaded soil. Fine scale temporal patterns may have been clearer and convergence may have been observed if abundances of each AM fungal isolate had been estimated from DGGE band brightness because abundances of AM fungal species can change dramatically over time (Merryweather and Fitter, 1998; Dumbrell et al., 2011; Hart et al., 2014).

Each *V. rossicum* plant was colonised by 7-18 AM fungal isolates (indicated by DGGE bands). Invasive plants may have a competitive advantage if they are able to be colonised by a range of AM isolates in the introduced range because they may differ from those with which they coevolved (Klironomos, 2002; van der Putten et al., 2007a; Pringle et al., 2009; Moora et al., 2011; Nuñez and Dickie, 2014). Furthermore, these novel symbiotic relationships could disproportionately benefit the invasive more than native species (i.e., the "enhanced mutualisms hypothesis", Reinhart and Callaway, 2006). Lekberg and Koide (2014) describe this as "bet-hedging", where it is beneficial for both plant and fungus to associate with multiple partners to optimise their benefits over time. A cross-continental study revealed that the invasive palm *Trachycarpus fortunei* was consistently colonised by a pool of geographically ubiquitous AM fungal isolates; however, there were also different unique isolates at each site (Moora et al., 2011). In contrast, some invasive plants appear to have more specific AM fungal requirements for invasion to occur. For example, a microcosm study found that invasion success of *Bidens pilosa* differed according to the identities of AM fungal isolates present (Stampe and Daehler, 2003). These idiosyncrasies between different plant species' requirements and responses to mutualist communities highlight the difficulties in determining generalised traits for plant invasiveness.

All sequenced DGGE bands were very closely related and within the Order Glomerales in Phylum Glomeromycota (Fig. 2.2; Krüger et al., 2012). It is possible that disturbance-tolerant AM fungi were inadvertently selected for by sieving the soil and growing them in artificial conditions (Antunes et al., 2006; Sýkorová et al., 2007) or by using primers that selectively amplify Glomerales (Mummey and Rillig, 2007; Krüger et al., 2012). However, a field study with *V. rossicum* targeting the small ribosomal subunit also found all AM fungi were in the Glomerales (Bongard et al., 2013).

In conclusion, this study with *V. rossicum* indicates that AM fungal communities may change in the presence of an invasive plant. The lack of convergence in composition between treatments over 29 weeks shows that the process of alteration of this mycorrhizal invasive plant on the AM fungal community extends further than the initial growing season, or that other factors are more important than plant host for determining AM fungal community composition. *Vincetoxicum rossicum* was colonised by many AM fungal species quickly and these generally remained in the plant throughout the 29 weeks of the study. This capacity to readily associate

with AM fungi could partly explain *V. rossicum*'s ability to successfully invade a wide range of habitats and soil types. Replicating this study over multiple sites and a range of environmental conditions would help us to understand whether the observed patterns that were consistent across two sites can be generalised. These results support that early eradication of invasive species to mitigate impacts on microbial communities may prevent positive feedback and increase the capacity to effectively restore invaded sites by minimising legacy effects.

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2.6. Supplementary material

Table S 2.1 Nutrient analysis of field soils from each location measured by University of Guelph Laboratory Services (http://www.guelphlabservices.com).

	Site 1		Site 2	
Soil variable	Uninvaded	Invaded	Uninvaded	Invaded
pH	8	7.8	8	7.9
CEC (Barium chloride method)	21.4	21.7	18	21.7
Total N (Combustion method; %)	0.15	0.2	0.09	0.22
Olsen P (mg/L)	8.7	11	3.7	8.7
K (ammonium acetate; mg/L)	70	75	56	68

Table S 2.2 Total number of quadrats each plant species occurred in at each location, of a total of 16 quadrats.

Plant species	Site 1		Site 2	
	Uninvaded	Invaded	Uninvaded	Invaded
Vincetoxicum rossicum		16		16
Poa pratensis	16	15	16	16
Hypericum perforatum	2	4	8	3
Linaria vulgaris	5	12	1	3
Vicia cracca	1	7	6	12
Solidago canadensis	15	14	16	
Cirsium arvense	5	1	1	
Euonymus sp.	1	1		
Lonicera tatarica	2	7		
Securigera varia	5	1		
Erigeron anuus			1	1
Taraxacum officinale			1	1
Symphyotrichum ericoides			1	1
Daucus carota		1	16	
Dactylis glomerata		2	2	
Potentilla recta		3	7	
Poaceae sp. 2		2	3	
Acer ginalla		2		
Vitis riparia		2		
Geum aleppicum		1		
Morus alba		1		
Rhus typhina		1		
Symphyotrichum ericoides		1		
Convovulus arvensis				3
Equisetum arvense			7	
Trifolium sp.			7	
Plantago lanceolata			7	
Plantago major			5	
Bromus sp.			2	
Poaceae sp.1			2	
Rhamnus frangula			2	
Equisetum palustre			1	
Solidago cf. *altissima*			1	
Total number of species	10	21	23	9

Table S 2.3 Results for 25 sequenced bands using the Basic Local Alignment Search (BLASTn) in Genbank.Table S3. Band numbers correspond to the band numbers in Fig. 2.2.

Band number	Closest relative (accession number)	Similarity (%)
3	*Glomus* sp. MUCL 43203 28S rRNA gene, strain MUCL 43203, clone 9 (AJ854592)	88
4	*Glomus aggregatum* isolate 08_34_32 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (JF439159)	83
5	*Funneliformis mosseae* clone Hsp38-15 28S ribosomal RNA (LSU) gene, partial sequence (JQ048904)	99
7	*Glomus lamellosum* isolate *G.lamellosum* Ex-Holotype-02 large subunit ribosomal RNA gene, partial sequence (AY541867) (current nomenclature: *Claroideoglomus lamellosum*)	96
11	*Glomus constrictum* isolate 08_48_12 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (JF439167) (current nomenclature: *Funneliformis constrictum*)	93
14	Uncultured *Glomus* clone Klon84 28S ribosomal RNA gene, partial sequence (JQ180466)	87
15	*Glomus* sp. MUCL 43207 28S rRNA gene, strain MUCL 43207, clone 11 (AJ854633)	94
17	Uncultured *Funneliformis* genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, clone GT025-23 (HF970338)	93
18	Uncultured Glomeromycota clone Mix_2E 28S large subunit ribosomal RNA gene, partial sequence (JF717544)	99
20	*Rhizophagus irregularis* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate from Poland (trap culture), clone pMK108-9 (FR750200)	86
21	*Funneliformis mosseae* clone Hsp38-15 28S ribosomal RNA (LSU) gene, partial sequence (JQ048904)	94
22	Uncultured Glomerales clone B08_04 large subunit ribosomal RNA gene, partial sequence (KC411226)	100
23	*Glomus* sp. Att690-23 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate Att690-23 (DAOM197198), clone pHS111-22 (FM992379)	99

Table S. 2.3 Continued.

Band number	Closest relative (accession number)	Similarity (%)
24	*Rhizophagus* cf. *irregularis* MUCL 43205 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate MUCL43205, clone pHS058-6 (FR750116)	92
25	*Glomus* sp. MUCL 43207 28S rRNA gene, strain MUCL 43207, clone 11 (AJ854633)	99
26	*Rhizophagus irregularis* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate Att857-12, clone pMK100-7 (FR750190)	99
27	*Rhizophagus* cf. *irregularis* FTRS203 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate FTRS203, clone pHS043-7 (FR750087)	91
28	*Rhizophagus irregularis* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate from Poland (trap culture), clone pMK108-9 (FR750200)	95
29	*Rhizophagus* cf. *irregularis* MUCL 43205 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate MUCL43205, clone pHS058-6 (FR750116)	98
30	*Glomus trimurales* isolate WA105 25S ribosomal RNA gene, partial sequence (FJ461859) (current nomenclature: *Diversispora trimurales*)	99
31	*Glomus* sp. MUCL 43205 28S rRNA gene, strain MUCL 43205, clone 6 (AJ854606)	92
32	*Glomus* sp. MUCL 43207 28S rRNA gene, strain MUCL 43207, clone 11 (AJ854633)	96
33	*Glomus claroideum* isolate RMF_133 28S ribosomal RNA, partial sequence (AF396785) (current nomenclature: *Claroideoglomus claroideum*)	99
34	*Claroideoglomus claroideum* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate Att1063-3/SW210, clone pHS035-33 (FR750074)	97
35	*Rhizophagus irregularis* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), isolate MUCL43195, clone pHS037-4 (FR750080)	98
37	*Glomus luteum* isolate SW202-9 clone 5 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (HQ857096) (current nomenclature: *Claroideoglomus luteum*)	100

Table S 2.4 Table showing the total number of plants of V. rossicum from each sampling time that each DGGE band was recorded, showing sites, treatments, and harvest times.

Site	1										2									
Treatment	U					I					U					I				
Harvest	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Band																				
1	1	2	1	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	2	1	0	3	2	0	0	0	1	1	0	0	0	0	0	1	1	0	2	0
3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
4	4	4	3	4	4	4	4	4	4	4	0	0	0	0	0	4	4	2	4	3
4a	0	0	0	0	0	0	0	3	4	2	0	0	0	0	0	0	0	0	0	0
5	0	2	3	4	1	4	2	2	4	3	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	1	0	3	4	4	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
9	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	3	0	1	2
10	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	4	0	1	2
11	0	0	0	0	0	0	0	0	0	0	2	2	0	1	0	0	0	0	0	0
12	2	1	2	1	4	0	1	1	0	0	0	3	0	1	3	0	0	0	0	0
12a	0	1	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
13	4	3	1	1	0	1	0	0	0	0	4	2	2	4	3	1	2	0	0	0
14	0	0	1	0	1	0	0	0	0	1	2	4	2	2	3	1	0	0	0	0
15	1	1	2	0	1	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1
16	0	0	0	0	1	0	0	1	0	0	2	3	3	1	1	0	0	1	2	2
17	1	0	2	0	3	0	1	1	3	1	1	0	1	0	1	0	0	1	0	0
18	0	3	3	0	4	4	4	4	4	4	4	3	2	2	1	3	4	4	4	4
19	1	3	0	1	4	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0
20	3	2	4	4	4	3	2	3	3	4	4	4	3	3	2	3	4	4	3	0
21	3	2	2	4	4	2	2	3	2	3	4	2	3	2	1	2	3	4	4	3
22	2	4	4	4	4	3	4	3	2	2	0	2	1	0	1	1	1	0	0	0
23	3	3	2	1	0	1	2	2	4	3	0	0	0	0	0	1	3	1	0	0
24	0	0	0	0	0	1	3	4	2	0	0	0	0	0	0	0	0	0	0	0
25	0	1	1	1	0	0	0	0	0	0	2	1	1	1	2	2	3	4	3	3
26	0	1	0	0	1	4	4	4	4	4	4	4	4	4	3	4	3	4	3	4
27	3	4	4	4	4	3	1	3	3	2	4	4	3	3	3	1	2	0	2	0
28	0	0	4	4	3	4	4	4	4	4	3	1	3	3	1	3	4	4	3	4
29	2	3	3	3	4	3	3	2	0	3	4	4	2	3	4	3	3	3	2	3
30	0	0	0	0	0	0	0	0	0	0	4	4	3	4	4	4	4	4	4	4
32	1	1	2	2	2	1	0	0	0	0	1	0	0	0	1	2	0	0	1	1

Table S 2.4 (continued)

Site	1										2									
Treatment	U					I					U					I				
Harvest	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Band																				
33	0	0	0	0	1	0	1	0	0	0	2	4	3	3	3	4	3	4	4	3
34	0	1	1	1	2	2	2	2	2	1	0	0	0	0	0	1	0	0	1	0
35	0	0	0	0	0	1	0	1	1	0	0	1	1	0	0	1	1	0	0	0
36	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
38	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	2	4	4
40	0	1	2	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
42	1	2	2	0	1	0	0	0	0	0	0	2	3	2	3	1	0	1	1	0
43	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	2	1	0
44	0	0	0	0	0	0	1	3	1	0	1	0	1	2	0	0	0	0	0	0

Chapter 3. Assessment of changes in pathogen and mutualist fungal communities and plant-soil feedbacks over 100 years of invasion

3.1. Abstract

One reason for success of invasive plants is thought to be the release from enemies that enable the plant to experience relatively higher growth and fitness in their invasive range. However, pathogens can subsequently accumulate over time and this may lead to declines in invasive populations. A few studies have shown that as invasion progresses over time, some invasive plants experience stronger negative plant-soil feedbacks (i.e., reduced growth in their own soil), thought to be due to accumulation of soilborne pathogens. However, the link between changes in soil microbial communities and plant-soil feedbacks over invasion time has not been demonstrated. Using a highly invasive plant in parts of eastern North America, *Vincetoxicum rossicum* (Apocynaceae), root-associated fungal communities and plant-soil feedbacks were investigated from 20 sites representing an invasion chronosequence of 0 to 100 years of invasion. Using data from high throughput sequencing of the internal transcribed spacer region of field-collected roots, I tested the hypothesis that roots from older invasions would have higher abundance and species richness of fungi that are known pathogens on other plant species compared to younger invasions. In a plant-soil feedback study in the glasshouse, I tested whether plants grown in soil from older invasions would have stronger negative feedbacks and enemy damage compared to soil from younger invasions or uninvaded sites. Roots of *V. rossicum* were colonised by a large number of fungal taxa, many of them that are known pathogens on other plant species or mutualistic arbuscular mycorrhizal fungi. However, there was no evidence of pathogen accumulation in terms of number, abundance, or species composition of fungi that are known pathogens on other plant species associated with *V.*

rossicum. In addition, there was no evidence for stronger negative feedbacks with invasion age, and *V. rossicum* consistently had high biomass when grown in live soil. These results indicate that although *V. rossicum* associates with many pathogens, this is not related to invasion time. In addition, *V. rossicum* gains a net benefit from all soil biota in its invaded range.

Keywords: pathogen accumulaton invasive decline hypothesis, plant-soil feedbacks, 454 pyrosequencing, invasion chronosequence, *Vincetoxicum rossicum.*

3.2. Introduction

Plants can alter soil microbial communities and in turn these communities may influence plant growth and fitness, known as plant-soil feedbacks (Bever, 1994; Bever et al., 2012). Positive feedback occurs when a given plant species has greater growth or fitness in its own “trained” soil compared to soil “trained” by other plant species, and is thought to be due to recruitment of microbial mutualists (Klironomos, 2002; Zhang et al., 2010; Bever et al., 2012). Conversely, negative feedback results in reductions in growth or fitness in a plant’s own “trained” soil, perhaps due to accumulation of host-specific pathogens (van der Putten et al., 1993; Klironomos, 2002; van de Voorde et al., 2012). These feedbacks can alter plant intra- and interspecific interactions in plant communities and therefore influence plant community structure (Bever et al., 2012; Pendergast et al., 2013; van der Putten et al., 2013). Invasive plants commonly show positive feedback in their invaded ranges and negative feedback in their home ranges, which is hypothesised to be due to enemy release and/or the accumulation of mutualists (Keane and Crawley, 2002; Callaway et al., 2004; Reinhart and Callaway, 2004; Blumenthal et al., 2009). However, the temporal dynamics of plant-soil feedback and associated changes in pathogen and mutualist communities is currently lacking.

Invasive plants can experience release from enemies when they invade (Keane and Crawley, 2002; Colautti et al. 2004). Over time, specialist pathogens may establish on invasive plants, which could lead to population declines (Bever, 2003; Mitchell and Power, 2003;

Simberloff and Gibbons, 2004; Eppinga et al., 2006; Hawkes, 2007; Orrock et al., 2012). Flory and Clay (2013) recently presented this as the “Pathogen Accumulation, Invasive Decline” hypothesis (PAID). Population declines due to pathogen accumulation could arise due to two non-mutually exclusive factors: through local adaptation of pathogens to the novel plant species over time (Gilbert and Parker, 2010), or through encountering a larger number of pathogenic agents as range expansion occurs (Mitchell and Power, 2003; Flory et al., 2011). An extensive analysis of all fungal and viral pathogens on 124 invasive plants in the USA indicated that both these processes have occurred since their introduction from Europe; plants that had been established for a longer period of time, had larger ranges, and had a history of agricultural use had a higher number of pathogens, irrespective of phylogeny (Mitchell et al., 2010). Empirical work focussing on soil biota and negative plant-soil feedbacks has demonstrated similar patterns. For example, negative feedback was stronger in 12 exotic plants that had established in New Zealand for longer periods of time than in those more recently established (Diez et al., 2010). Dostál et al. (2013) found that soil from sites of older invasion of *Heracleum mantegazzianum* reduced its growth more than soil from younger invasions. However, in both studies, associated changes in soil biota and the potential pathogens driving these patterns are unclear.

While pathogen accumulation on invasive plants has been demonstrated, concurrent declines in growth or fitness of the invader are rarely observed (Hawkes, 2007; Flory and Clay, 2013). One possibility for this could be because mutualists can ameliorate the negative effects of high pathogen loads (Morris et al., 2007). For example, arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) are considered to be mutualists (Hoeksema et al., 2010) and have been shown to reduce the effects of pathogens in multiple plant species (Newsham et al., 1995; Borowicz, 2001; Maherali and Klironomos, 2007; Sikes et al., 2009; Lewandowski et al., 2013). Taxa within the Glomeraceae family are particularly good for pathogen protection (Maherali and Klironomos, 2007; Sikes et al., 2009). The mechanism behind this is unclear, but it could be due to spatial exclusion in the root niche, enhanced nutrient uptake, or a combination of factors (Maherali and Klironomos, 2007; Smith and Read, 2008; Sikes et al., 2009). Therefore, if invasive plants accumulate pathogens over time, they may also accumulate these pathogen-protecting AM fungi as well, which could result in an overall neutral growth effect.

These ideas of feedback and pathogen accumulation in invasive plants were combined by investigating long term dynamics in plant-soil feedbacks and root-associated fungal communities in a highly invasive exotic plant in eastern North America, *Vincetoxicum rossicum* (Apocynaceae; Douglass et al., 2009). In southern Ontario, this plant often invades areas of *Solidago* sp. (Asteraceae) and *Pinus* (Pinaceae; Cappuccino, 2004; DiTommaso et al., 2005a; Kricsfalusy and Miller, 2010; Anderson 2012; Bongard et al., 2013). *Vincetoxicum rossicum* has been shown to experience enemy release in Ontario (Agrawal et al. 2005) and also associates with a large number of AM and non-AM fungi (Bongard and Fulthorpe, 2013; Bongard et al., 2013; this thesis chapter 2). The overall objective of this study was to investigate whether *V. rossicum* experiences pathogen accumulation and test if negative feedback became stronger as invasion progressed through time. Similar to Dostál et al. (2013), I used a space-for-time substitution approach and constructed an "invasion chronosequence". I conducted two experiments using plants and soil collected from multiple sites where first records of *V. rossicum* were from one to approximately 100 years of invasion. First, high throughput sequencing (454 pyrosequencing) was used to determine the species composition of root-associated fungal communities in *V. rossicum* along the invasion chronosequence. I expected there to be differences in composition of root-associated fungi on *V. rossicum* from sites of different invasion ages. In particular, I expected to find higher species richness and abundance of fungi that are known pathogens on other plant species in the older compared to younger invasion ages. Since *V. rossicum* rapidly associates with many AM fungi (this thesis chapter 2), I did not expect a change in AM fungal species richness in different invasion ages. However, I expected there to be a change in AM fungal species composition towards those associated with pathogen protection, such as those within Glomeraceae (Maherali and Klironomos, 2007; Sikes et al., 2009; Wehner et al., 2010).

In a concurrent plant-soil feedback experiment, I tested the hypothesis that *V. rossicum* grown in soil from recently invaded sites would experience positive feedback, but negative feedback and evidence for pathogen damage would occur in plants grown in soil from older invaded sites. Specifically, I expected that *V. rossicum* plants growing in soil from older invasion ages would have lower biomass and fitness than those growing in uninvaded soil or in soil from young invasion ages. I further expected higher colonisation of AM and non-AM fungi in older invasion ages, reflecting the pathogen protecting function of AM fungi and accumulated pathogens (Smith and Read, 2008; Wehner et al., 2010).

3.3. Methods

3.3.1. Characterising root-associated fungal communities along an invasion chronosequence

3.3.1.1. Sample collection and DNA extraction

Root-asssociated fungal communities associated with *V. rossicum* at sites of different invasion ages were assessed by 454 pyrosequencing using field-collected roots. Invasion ages ranged from the early 1900s to 2012 and were determined based on the first record of *V. rossicum* ator near the site from a combination of literature, herbarium records, and information from local land managers and conservation authorities (Table S 3.1). In 2013, roots were collected from four plants at five sites for each invasion age: old (Fig. 3.1; *V. rossicum* first recorded approximately 100 years ago), intermediate (50-60 years), and young (<12 years), for a total of 15 sites and 60 root samples. All sites, including those within the City of Toronto, were in natural areas and had experienced minimal or no management for invasive species eradication. At each site, four *V. rossicum* plants within 5 m of each other were excavated with a spade and placed in separate plastic bags. Plants were refrigerated at 4˚C at the end of each day. Root samples were taken from each plant from crowns of similar sizes, washed in tap water and then surface disinfected in 70% ethanol for 3 minutes, followed by 10 minutes in 1% sodium hypochlorite, and three rinses in sterile water. Roots were patted dry with sterile paper towels and stored in sterile 2 ml microcentrifuge tubes at -20ºC until DNA extraction. Previous work had shown this to be an effective surface disinfection method (i.e., no fungal growth on media that was swiped with disinfected root surface; this thesis chapter 4). The surface disinfection was important to eliminate saprobes, especially because many fungi function as saprobes in the soil but can become pathogenic in roots (Agrios, 2005).

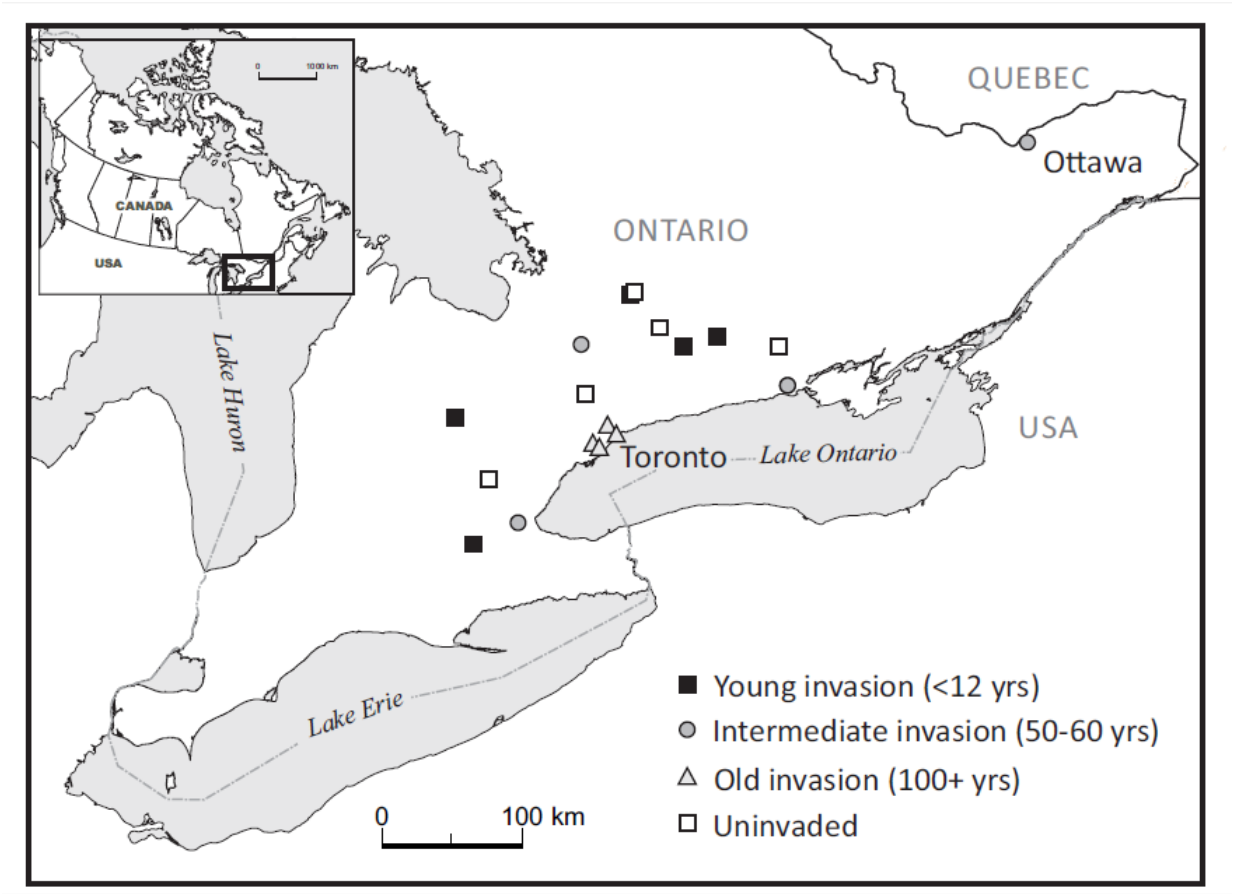

Fig. 3.1 Map of 20 sites representing different invasion ages of *V. rossicum* in southern Ontario, Canada. Each invasion age was represented by five replicate sites. Root samples for 454 pyrosequencing were collected from the young, intermediate, and old invasion ages (n=15). Soil for the plant-soil feedback study was collected from sites of all invasion ages, including the uninvaded sites (n=20).

For DNA extraction, frozen roots were homogenised by placing four sterilised 3 mm tungsten carbide beads into each microcentrifuge tube with the roots, immersing in liquid nitrogen for 30 seconds, and shaking at 30 mHz for one minute in a MoBio 96 Well Plate Shaker (MoBio Laboratories, Carlsbad, CA, USA). These steps were repeated a total of 12 times to ensure sufficient root homogenisation. Roots did not thaw during this process. DNA was extracted with the Macherey-Nagel Nuceleospin Mini kit using CTAB cell lysis buffer (Macherey-Nagel and Co. KG, Düren, Germany). Beads were sterilised between extractions by washing with deionised water, soaking in UltraClean Lab Cleaner to remove nucleases and nucleic acids (MoBio Laboratories, Carlsbad, CA, USA), rinsing thoroughly, and autoclaving at 121ºC and 18

psi for 15 minutes. DNA from two technical replicates was pooled, purified, and stored at -20°C. Two replicates from one site of old invasion age were lost due to a labelling error, resulting in a total of 58 plant DNA samples.

3.3.1.2. 454 library preparation and sequencing

Purified DNA was sent to McGill University and Génome Québec Innovation Centre for 454 library preparation and sequencing (Montréal, QC, Canada) using fungal-specific primers ITS1F (CTTGGTCATTTAGAGGAAGTAA; Gardes and Bruns, 1993) and ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990). These primers are specific to the rDNA Internal Transcribed Spacer (ITS) region of fungi and, crucially, do not amplify plant DNA (Bellemain et al., 2010). A nested PCR approach was used to prepare the samples for 454. The first PCR was to initially amplify fungal DNA and the second PCR was to add the extended primers with 454 adapters and molecular identification (MID) tags (Lindahl et al., 2013).

All PCRs were run using the Faststart High Fidelity PCR System from Roche. The initial PCR to amplify fungi was run in 5 µl volumes: 4.5 mM magnesium chloride, 0.2 mM deoxyribonucleotide triphosphate 100 nM of each primer 1 µl DNA, 0.25 U taq polymerase, 0.5 µl 10X buffer, 0.25 µl dimethyl sulfoxide (DMSO), made up to 5 µl with sterile DNAse-free water. The PCR program was as follows: initial denaturation at 95˚C for 15 minutes, followed by 35 cycles of 95˚C for 20 seconds, annealing at 60˚C for 30 seconds, and elongation at 72˚C for 45 seconds, followed by a final elongation step at 72˚C for 5 minutes. The second PCR to incorporate the MID tags was run in a total volume of 20 µl, with 4.5 mM magnesium chloride, 0.2 mM deoxyribonucleotide triphosphate 100 nM each primer 1 U taq polymerase 2 µl 10X buffer 1 µl DMSO, and template DNA from the first PCR diluted 1:100. The PCR program was slightly modified: initial denaturation at 95˚C for 10 minutes, followed by 15 cycles at 95˚C for 15 seconds, annealing at 68˚C for 30 seconds, and elongation at 72˚C for 60 seconds, followed by a final elongation step at 72˚C for 3 minutes. DNA concentrations were measured by picogreen and standardised to 50 ng per sample prior to sequencing on a Roche 454 GS FLX+ sequencer.

3.3.1.3. Bioinformatics processing

Bioinformatics processing of 454 sequences was performed by McGill University and Génome Québec Innovation Centre (Montréal, QC, Canada). A total of 661,021 reads were obtained. All 454 sequencing adapters were removed and reads were trimmed to 350 bp. Reads shorter than 350 bases were discarded, leaving a total of 660,643 reads. Eight more reads containing known 454 contaminants (adapters) were filtered out using the DUK software (http://duk.sourceforge.net/). Remaining reads with average quality scores less than 30, more than 10 bases that could not be determined (Ns), or having 10 nucelotides below quality 15 were discarded. Remaining reads were clustered at 100% similarity, followed by clustering at 99% in dnaclust v.3 (Ghodsi et al., 2011) similarity (denoising). Clusters with fewer than three sequences were discarded and chimeras were removed using UCHIME denovo followed by UCHIME reference (Edgar et al., 2011). Resulting clusters were clustered once more at 97% similarity in dnaclust v.3 (Ghodsi et al., 2011). After removing clusters that had fewer than three sequences, there were a total of 409,859 reads packed in 865 clusters. The resulting operational taxonomic units (OTUs) were assigned to taxonomic lineages by classifying each cluster with the Ribosomal Database Project (RDP) classifier v. 2.5 with 100 bootstraps (Wang et al., 2007) using a UNITE ITS training set (http://unite.ut.ee/sh_files/sh_qiime_release_13.05.2014.zip; Kõljalg et al., 2013) to which additional plant and eukaryote outlier ITS sequences had been added. Taxonomic names were assigned at each taxonomic level where RDP classifier confidence values were greater than 0.50. This means that the cluster was assigned to that taxonomic group in at least 50 of the 100 bootstraps (Wang et al., 2007). Taxonomic labels below genus were not assigned to OTUs due to the relatively short sequences that make it difficult to accurately delineate to species level. OTUs were labelled by their OTU numbers and a unique number to be able to identify between OTUs in the same taxa that were different at 97%. Rare OTUs that occurred in only one of the 58 samples were removed to further denoise the data (McCune and Grace, 2002; Legendre and Legendre, 2012).

3.3.1.4. Determining pathogens and mutualists

To investigate if there was evidence for accumulation of pathogens on *V. rossicum* at sites of older invasion, all OTUs that were identified to genus were classified according to whether they were a plant pathogen; a genus with at least one species identified as a plant pathogen in the literature was considered a potential pathogen on *V. rossicum*. I searched for occurrences of fungi that are known pathogens on other plant species in two ways. Initially, I searched the fourth edition of the *Names of Plant Diseases in Canada* book (Couture et al., 2003) for records of each genus as a pathogen. For genera that were not recorded in this book, I searched the Web of Science database in July 2014 using the following search terms for each genus: "genus name" AND pathog*, so that the results would include all possible terms such as pathogen, pathogenicity, and pathogenic. Investigating each record listed indicated whether each genus included a potential pathogen. To investigate changes in mutualistic taxa, OTUs that were defined to genus and were in Phylum Glomeromycota were classified as AM fungi (Schussler et al., 2001; Smith and Read, 2008; www.mycobank.org).

3.3.1.5. Data Analysis

All statistical analyses were performed in R version 3.0.2 (R Core Development Team, 2014) using packages where specified. OTU composition of the root-associated fungi for all 58 plant samples and OTUs at 97% similarity were visualised using a principal coordinates analysis (PCoA). The PCoA was run in 6 dimensions using function 'cmdscale' in base R and OTU abundances, in terms of number of reads. Bray-Curtis distance was square-root transformed to avoid negative eigenvalues (Legendre and Legendre, 2012). The advantage of PCoA is that there is no underlying assumption of linearity between species distributions and the environment (McCune and Grace, 2002). A permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) with Bray-Curtis distance was run to investigate significant predictors of root-associated fungal community composition. The only assumption for PERMANOVA is that observations are independent (Anderson, 2001). Predictors were invasion age, soil ammonium (mg per g dry soil), nitrate (mg per g dry soil), phosphorus (µg per g dry soil), and pH. These soil parameters were the least correlated with each other (data not shown) and are important for

plant growth (Foth, 1990). Site was a random effect and *P*-values were estimated from 1000 randomisations using the 'adonis' function in vegan (Oksanen et al., 2013b).

3.3.1.5.1. Pathogen and mutualist analyses

Pathogen accumulation could be reflected either as an increase in the number of pathogen OTUs (richness), or in the abundance of pathogens in terms of the number of sequence reads. Two generalised linear mixed models were run using function "glmmPQL" in package MASS (Venables and Ripley, 2002), with invasion age as a categorical predictor variable and site as the random effect. This was to account for overdispersion, because the assumption of equal variances was not met. Other assumptions of generalised linear mixed models were met: independence of within-group errors and normal distribution of random effects. To investigate if older invasion ages had a higher number of pathogens I compared pathogen richness in different ages specifying a Poisson response. Abundance data were converted to a proportion to compare between samples that had different total numbers of reads by calculating the number of reads of potential pathogens divided by the total number of reads per sample. This proportion was specified as a binomial response. Pre-planned contrasts were specified for both models: contrast 1: young compared to the mean of the intermediate and old invasion ages combined; contrast 2: intermediate compared to old invasion age. Pre-planned contrasts allow the user to specify contrasts between means of groups of interest and are more powerful than post-hoc tests (Crawley, 2002). A PERMANOVA was run to investigate significance of invasion age, soil ammonium, nitrate, phosphorus, and pH, using the same methods as for the entire root-associated fungal community (Oksanen et al., 2013b). Analyses for investigating differences in AM fungal richness, proportion, and changes in composition were the same as for the pathogen analysis.

3.3.2. Plant-soil feedbacks along an invasion chronosequence

3.3.2.1. Experimental set up

A second study was performed to assess effects of soil microbial communities along the invasion chronosequence on *V. rossicum* growth. A plant-soil feedback approach was used, where soil from different invasion ages had been 'trained' by *V. rossicum* for different periods of

time in the field. Soil was collected from the five sites for each of the old, intermediate, and young invasion ages used above, as well as five sites that had no record of invasion by *V. rossicum* (uninvaded; Table S 3.1), for a total of 20 sites (Fig. 3.1). The uninvaded sites had no record of ever being invaded by *V. rossicum* but were in areas that would be expected for *V. rossicum* to invade, i.e., areas of *Solidago canadensis* or *Pinus resinosa* (Cappuccino, 2004; DiTommaso et al., 2005a; Miller et al., 2007; Kricsfalusy and Miller, 2010; Anderson 2012; Bongard et al., 2013). Soil nutrient tests confirmed that values of ammonium, total nitrogen, nitrate, carbon, phosphorus and pH from the uninvaded sites were all within the same ranges as those of the invaded sites (Fig. S 3.1). At each site, soil was collected from an area of approximately 3 m^2 that was at least 4 m from the nearest trail or road. Soil and root material were collected to a depth of approximately 30 cm and placed in an opaque plastic bag for transport. All soil samples were refrigerated at the end of each day. Soil was collected using aseptic field techniques to avoid cross contamination between sites, where spades were thoroughly scrubbed with undiluted household bleach and rinsed with water between sites.

Soil from each site was homogenised by passing through a 4 mm sieve. To prevent cross-contamination, sieves were soaked in a diluted bleach solution for a minimum of 20 minutes and all benches were bleached and rinsed between samples. Rubber gloves were worn and were changed between samples. Approximately 18 L of soil were sieved from each site; 9 L were kept as live inoculum and 9 L kept for sterilisation. Roots are important sources of inoculum for some root fungi (e.g., AM fungi; Tommerup and Abbot, 1981; Klironomos and Hart, 2002). To account for this, roots from each site were collected while sieving and cut into 2-3 cm pieces using flame-sterilised scissors. Then 25 g roots were mixed into live sieved soil and another 25 g were mixed into the soil for sterilisation. The roots for invaded sites were composed entirely of *V. rossicum*, while roots from the uninvaded sites were composed of a mixture of the plants present in the sample. The soil for sterilisation was autoclaved at 121ºC for 90 minutes, left at room temperature for one day, and then autoclaved again. Sterilised soil was stored at room temperature for 10 days prior to potting to allow mineralisation. Live soil was stored at 4ºC to mitigate changes in microbial communities.

There were seven replicate pots for each of the 20 sites, with an additional seven sterile controls, making a total of 147 2.8 L pots. All equipment was disinfected as when sieving. Pots were lined with mesh and placed on saucers to prevent substrate and nutrient loss. Each pot

contained a substrate mixture comprised of equal volumes of sterile sand (non-calcareous “B” sand, Hutcheson Sand and Mixes, Huntsville, ON, Canada), turface (calcined, non-swelling illite and silica clay, Turface Athletics MVP, Profile Products LLC, Buffalo Grove, IL, USA), live soil from that site, and a mix of sterile soil from the other sites. For example, each pot for Site A contained 700 ml sand, 700 ml turface, 700 ml live soil from Site A and 700 ml equal mix of sterile soil from all other sites. Control pots did not contain live soil. The sterile soil was added to minimise differences in soil nutrients, texture, and organic matter (Bever, 1994; Pendergast et al., 2013). A 1 cm layer of sterile sand was placed on the surface to prevent cross-contamination through airborne particles.

Seeds collected from one site in Toronto were stratified at 4ºC between moist sheets of filter paper for three weeks prior to planting. Five weeks prior to potting the experiment, seeds were surface disinfected for 3 minutes in 70% ethanol followed by 10 minutes in 1% sodium hypochlorite and three rinses in sterile water. They were then pre-germinated in a tray with an equal mix of sterile sand, turface and soil (autoclaved twice at 121ºC for 90 minutes). One pre-germinated seedling was planted into each pot using bleach-disinfected equipment. Plants that did not survive the transplant were replaced within one week (11 of 148 plants). Additional plants that grew in the soil were clipped at the surface to ensure there was only one *V. rossicum* plant growing in each pot. Plants were watered with reverse osmosis water between two and seven days each week over the course of the experiment and under a 14/10 day/night photoperiod with temperature ranging between 20 and 24ºC. Pots were re-randomised monthly. A low concentration of slow release fertiliser was added to each pot five weeks after potting (1 g Nutricote 13:13:13 type 100 = 0.4 ppm NPK, Plant Products, Brampton, Ontario, Canada).

The experiment was harvested after 12 weeks and 133 experimental units survived the course of the experiment. This resulted in a final dataset with 31 units for each of intermediate and old invasion age, and 32 units for uninvaded and young invasion age, and seven controls. These mortalities were therefore not distributed in any pattern in relation to invasion age. One replicate died from each of three sites in the uninvaded and young invasion age. Four replicates died from one site in the intermediate invasion age. For the old invasion age, one replicate died from each of two sites and two replicates were lost from one site. No plants died in the sterile controls.

At harvest, shoots were separated from roots and washed in tap water. Roots were thoroughly washed in tap water, dried with a paper towel and weighed to obtain fresh weight. Two subsamples of roots were collected from different parts of the root and stored in tissue cassettes in 70% ethanol for later determination of fungal colonisation and pathogen damage. The roots were then re-weighed to obtain fresh weight after subsamples were taken to be able to back-calculate total root biomass. Shoots and roots were stored in separate paper bags and dried at 60˚C for at least three days before being weighed. Dry weights were measured twice to ensure consistency and accuracy.

3.3.2.2. Estimating root damage and AM fungal colonisation

To gain a representative sample of root damage from each site, three randomly selected pots per site and three controls were used to assess root damage caused by pathogens and other soil biota such as nematodes. Roots were rinsed under tap water to remove ethanol and cleared by placing in 10% potassium hydroxide for 90 minutes at 90˚C then mounted in 50% glycerol. Signs of root decay or discolouration and/or chew marks were recorded at 200X magnification within the entire field of view (Reininger et al., submitted).

To gain a representative sample of AM and non-AM fungal colonisation from each site, three randomly selected pots per site and three controls were used to estimate percent root colonisation. These were cleared as for the pathogen assessment, rinsed, and placed in a 5% ink-vinegar solution for 10 minutes at 90˚C (Pelikan black ink; Vierheilig et al., 1998). Destaining was done by placing the roots in 50% glycerol for a minimum of three hours before mounting on to slides. Percent colonisation was measured using the grid intersection technique with 100 intersections at 200X magnification (McGonigle et al., 1990). In each intersection I recorded the presence of AM fungi, arbuscules, vesicles, and non-AM fungi. All slides for assessing pathogen damage and fungal colonisation were assigned randomised numbers to avoid potential treatment bias when measuring.

3.3.2.3. Data analysis

All data were analysed using R v. 3.0.2 (R Core Development Team, 2014). If *V. rossicum* experienced overall positive feedback then I expected biomass to be lower in the sterile control soil compared to the live soil. To initially test for feedback, I ran a general linear mixed model, with site as a random effect using function 'lme' in package nlme (Pinheiro et al., 2014). Total biomass was the response variable and invasion age was the predictor variable. Pre-planned contrasts were specified: contrast 1: sterile control compared to the mean of live soil combined (uninvaded and all invasion ages); contrast 2: uninvaded compared to the mean of young, intermediate, and old invasion ages combined; contrast 3: young compared to the mean of the intermediate and old invasion ages combined; contrast 4: intermediate compared to old invasion age. A separate model was run with root-shoot ratio as the response. A generalised linear model was run using the number of flowers and seed pods per plant as the response (Poisson link). This model was run using function "glmmPQL" in package MASS (Venables and Ripley, 2002), which uses penalised quasi likelihood to account for overdispersion that was evident from the initial model run in lme4 (Bates et al., 2014).

Separate generalised linear mixed models were run to investigate the relationship between root damage and invasion age. The proportion of intersections with pathogen damage was modelled as a binomial response using "glmmPQL" in package MASS (Venables and Ripley, 2002). The pre-planned contrasts were the same as for the biomass data. The relationships between colonisation by AM and non-AM fungi and invasion age were run against four response variables: proportion of intersections with AM fungi (including hyphae, arbuscules, and vesicles), arbuscules only, vesicles only, and non-AM fungi. Sterile controls were omitted from these analyses because there were no signs of colonisation by AM or non-AM fungi, so the following pre-planned contrasts were specified: contrast 1: uninvaded compared to the mean of young, intermediate, and old invasion ages combined; contrast 2: young compared to the mean of the intermediate and old invasion ages combined; contrast 3: intermediate compared to old invasion age.

3.4. Results

3.4.1. Characterising root-associated fungal communities along an invasion chronosequence

Only fungal sequences were detected in the 58 root samples from 15 sites of three invasion ages of *V. rossicum* using 454 pyrosequencing with primers ITS1F and ITS4. The final dataset comprised 406,684 reads clustered into 478 OTUs at 97% similarity that occurred in at least two of the 58 plant samples. Each root sample had a mean of 7,012 reads (range: 2,299-11,995) and 49 OTUs (range: 7-83). Each OTU was represented by a mean of 851 reads (range: 3-82,888) and occurred in a mean of six plants (range: 2-57). Most (305) occurred in less than five root samples, and only three OTUs occurred in more than 35 samples: Cyanonectria.133889 was present in 52 samples, Plectosphaerella.177525 in 55 samples, and Phycomyces.234073 was in 57 samples. Four phyla were detected in the OTUs: 238 OTUs belonged to the Glomeromycota (1183 reads) 183 to Ascomycota (1351 reads) 15 to Basidiomycota (50 reads), and eight to Zygomycota (95 reads). The remaining 34 OTUs (133 reads) were unable to be assigned to phyla based on the RDP classification with a confidence >0.5. A total of 23 Orders and 48 Families were represented, although 68 and 86 OTUs were unable to be assigned to Order or Family respectively (Tables S 3.2 and S 3.3). In terms of the proportion of reads, Orders Helotiales and Pezizales were most abundant in the young invasion age, Pleosporales was most abundant in the intermediate invasion age, and Glomerales was most abundant in the old invasion age (Fig. 3.2). Agaricales and Chaetothyriales were more abundant in young and intermediate invasion age (Fig. 3.2).

Using abundance data for all 478 OTUs at 97% similarity across the 58 plant samples, the PCoA explained 36.4% of the total variation in root-associated fungal OTU composition. Samples did not cluster in ordination space according to invasion age (Fig. 3.3). Samples in the top right of the PCoA plot of site scores were characterised by abundant Helotiaceae.238153, Helotiaceae.131560, Plectosphaerella.32872, and Paraglomus.225843, but these were not related to particular sites or invasion ages. This was confirmed by the PERMANOVA because invasion age was not a significant predictor of root-associated fungal composition. The soil parameters were not also significant predictors (Table 3.1).

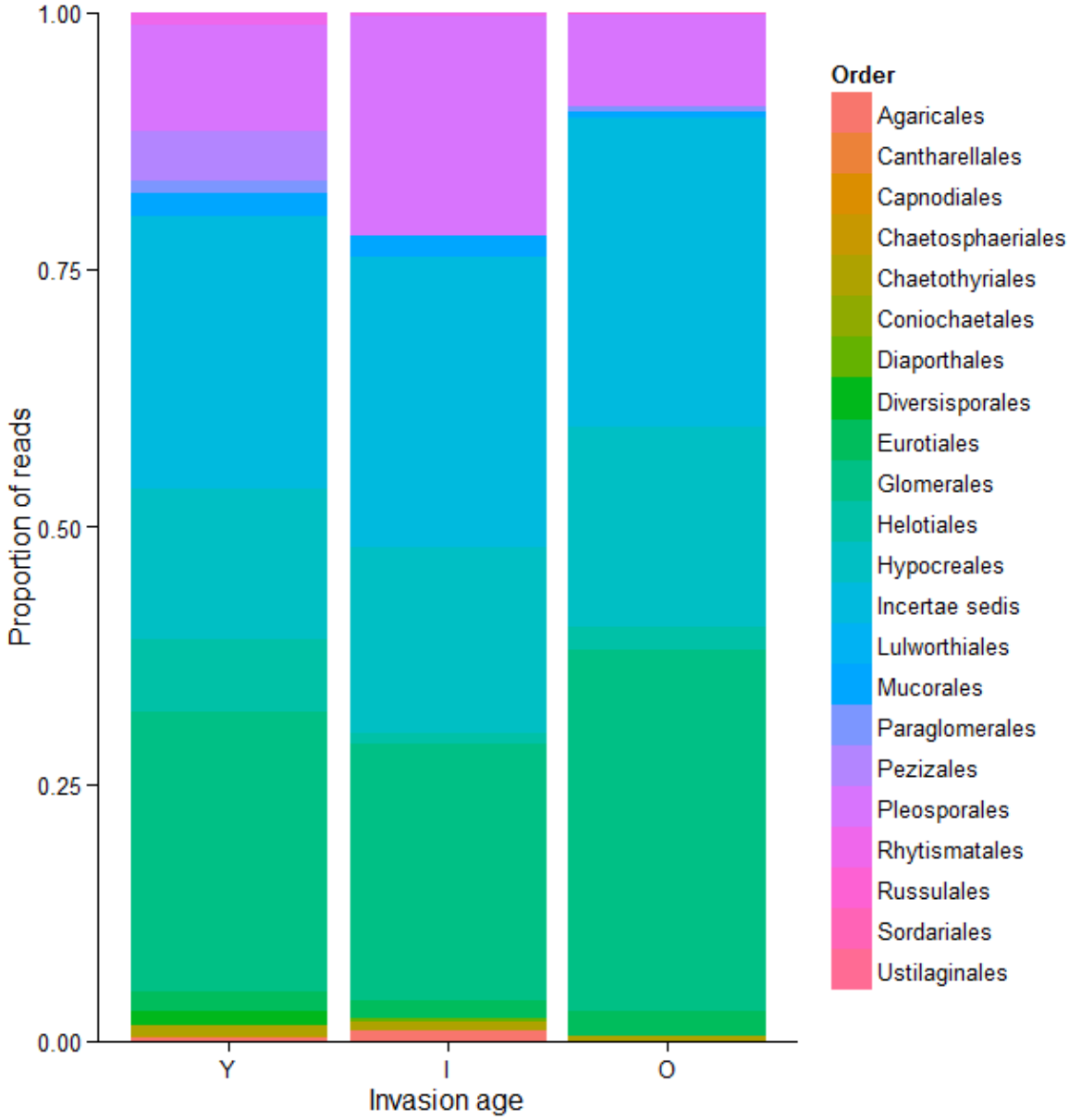

Fig. 3.2 Proportion of reads of each fungal Order from 454 pyrosequencing for 478 fungal OTUs identified from roots of 58 *V. rossicum* plants from 15 sites in three invasion ages. Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). The graph was produced in R using package 'ggplot2' (Wickham 2009).

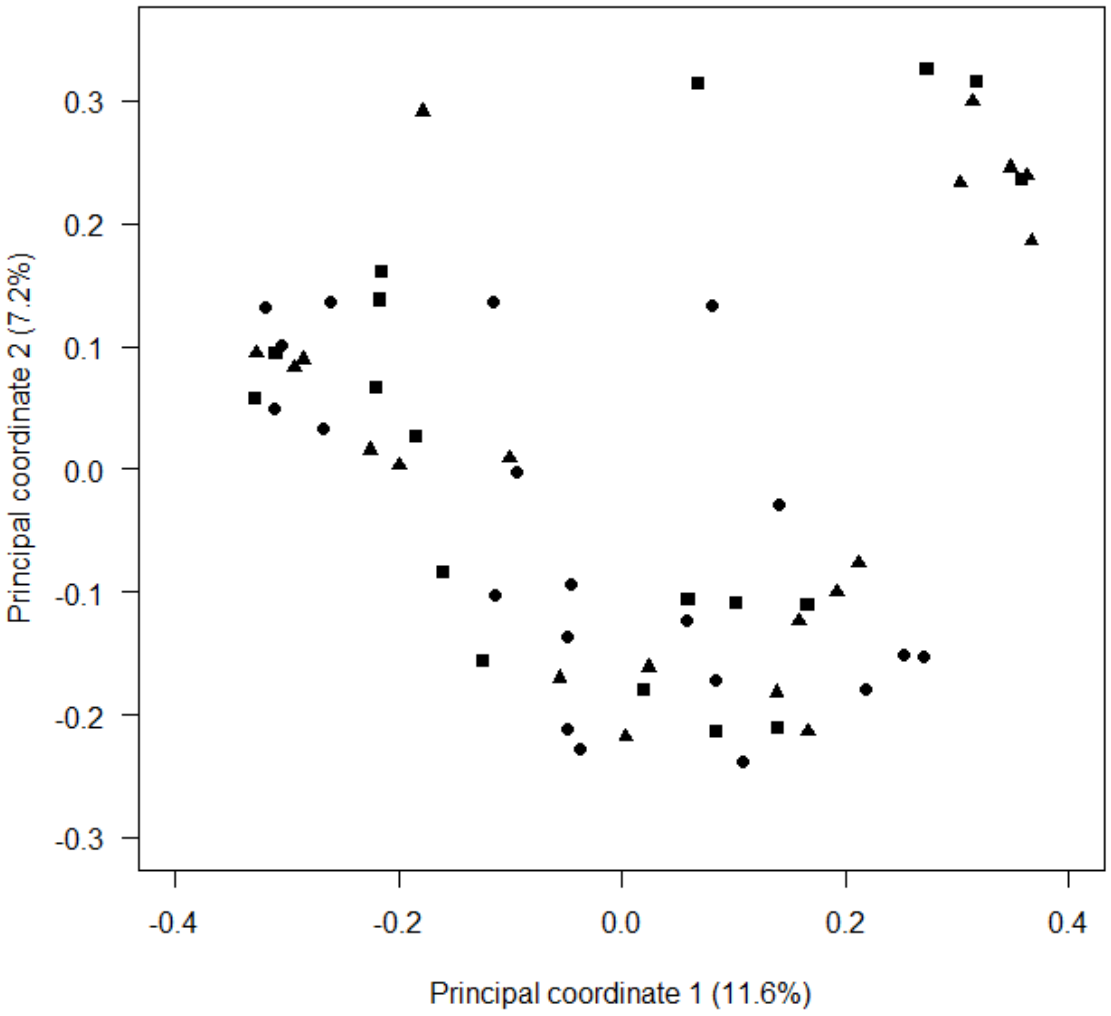

Fig. 3.3 Site scores of principal co-ordinates analysis (PCoA) using square-root transformed Bray-Curtis distance on abundance data for 478 fungal OTUs identified from roots of 58 *V. rossicum* plants from 15 sites in three invasion ages. Squares = young invasion (<12 years), circles = intermediate invasion (50-60 years), and triangles = old invasion age (100+ years). Values in brackets on axes correspond to the amount of variation explained by each axis (total explained in 6 dimensions = 36.4%).

Table 3.1 Results from permutational analysis of variance (PERMANOVA) testing potential predictors of root-associated fungal community composition from 454 pyrosequencing of 58 root samples of *V. rossicum* from 15 sites in three invasion ages: young (<12 years), intermediate (50-60 years), and old (100+ years).

Variable	Variation explained (%)	df	SS	MS	Pseudo *F*	*P*
Invasion age	3.72	2	0.70	0.35	1.08	0.390
Soil ammonium	1.83	1	0.34	0.34	1.07	0.182
Soil nitrate	2.49	1	0.47	0.47	1.45	0.712
Soil phosphorus	1.56	1	0.29	0.29	0.91	0.725
Soil pH	2.97	1	0.56	0.56	1.73	0.173
Residuals	87.43	51	16.49	0.32		
Total	100	27	18.86			

3.4.1.1. Was there accumulation of pathogens?

Of the 478 unique OTUs, 351 were identified to genus, falling into 62 fungal genera (i.e., each genus was represented by multiple species at 97% similarity; Table S 3.4). The literature search showed that 32 of these 62 genera contained at least one species recorded as a plant pathogen (Table S 3.4). However, the generalised linear mixed models showed no evidence for greater abundance or richness of fungi that are known pathogens on other plant species on *V. rossicum* at older invaded sites (Table 3.2, Fig. 3.4). Invasion age and the soil parameters were not significant predictors of the composition of fungi that are known pathogens on other plant species (Table 3.3).

3.4.1.2. Were there changes in mutualist communities?

Of the 62 genera, eight were AM fungi (Table S 3.4). Only one root sample lacked AM fungal OTUs and it was from young invasion age. There was no evidence for plants from different invasion ages to have different richness or abundance of AM fungi (Table 3.4, Fig. 3.4).

Invasion age and the soil parameters were not significant predictors of AM fungal composition (Table 3.5).

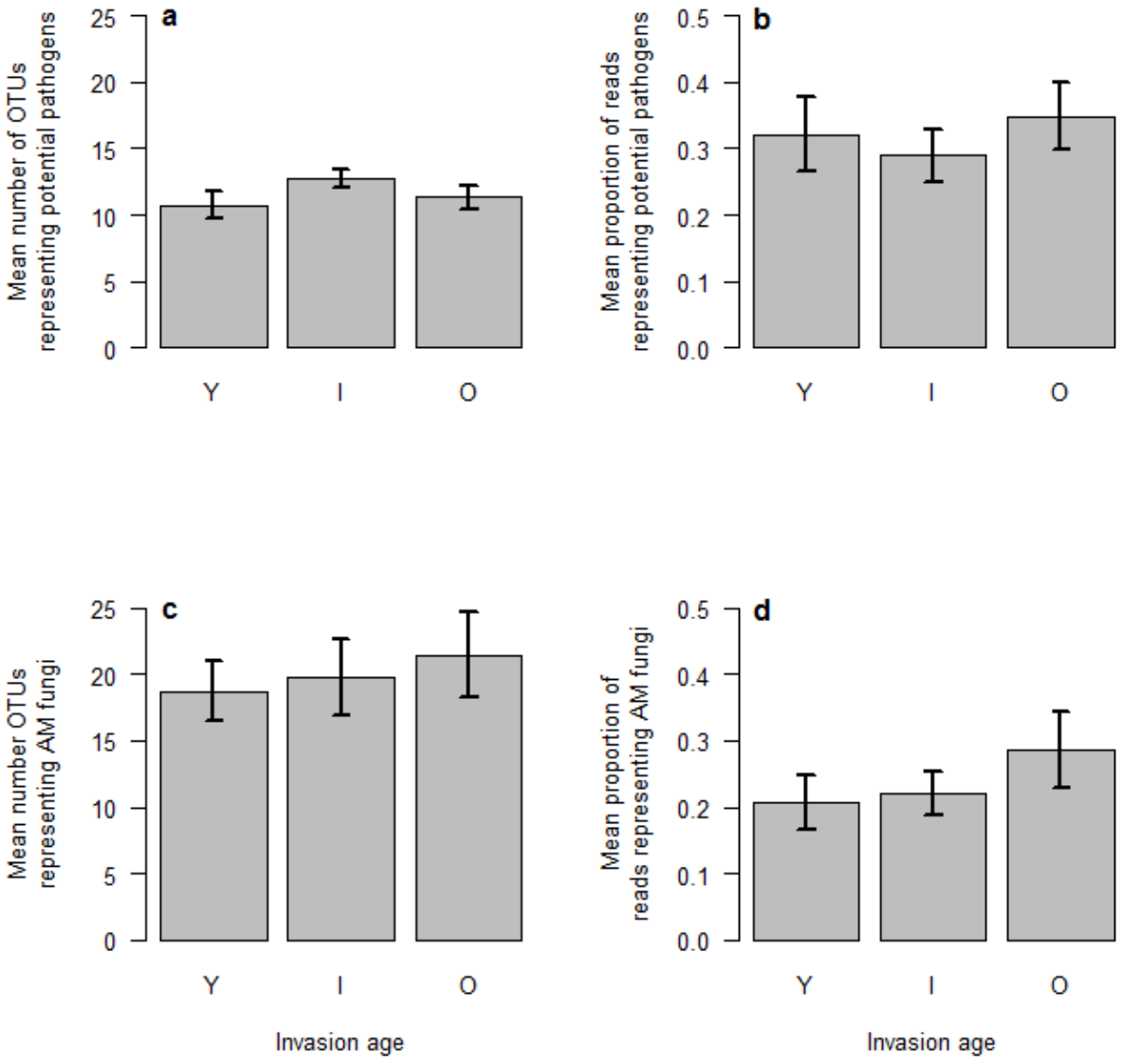

Fig. 3.4 Groups of root-associated fungi in *V. rossicum* showing the (a) mean number of OTUs of potential pathogens, (b) mean proportion of reads of potential pathogens, (c) mean number of OTUs of AM fungi, (d) mean proportion of reads of AM fungi, from 58 root samples from 15 sites of different invasion ages. Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). Potential pathogens are those that are known pathogens on other plant species.

Table 3.2 Coefficient estimates, *t*-statistics, and *P* values for each fixed effect contrast in generalised linear mixed models for potential fungal pathogens from roots of *V. rossicum* from 15 sites in three invasion ages: young (<12 years), intermediate (50-60 years), and old (100+ years). Potential pathogen species richness was a Poisson response. Potential pathogen abundance was a binomial response as the proportion of reads of potential pathogens. Site was a random effect. Standard errors are in brackets. Potential pathogens are those that are known pathogens on other plant species.

Model	Contrast	Coefficient (standard error)	*t*	*P*
Pathogen species richness	Intercept	2.45 (0.04)	55.09	0.000
	Contrast 1	-0.04 (0.03)	-1.20	0.252
	Contrast 2	0.06 (0.05)	1.06	0.312
Pathogen abundance	Intercept	-0.79 (0.15)	-5.14	0.000
	Contrast 1	-0.03 (0.11)	-0.23	0.820
	Contrast 2	-0.16 (0.19)	-0.84	0.417

Note: Pre-planned contrasts: contrast 1: young compared to the mean of the intermediate and old invasion ages combined; contrast 2: intermediate compared to old invasion age.

Table 3.3 Results from permutational analysis of variance (PERMANOVA) with potential predictors of potential fungal pathogen community composition from 454 pyrosequencing of 58 root samples of *V. rossicum* from 15 sites in three invasion ages: young (<12 years), intermediate (50-60 years), and old (100+ years). Potential pathogens are those that are known pathogens on other plant species.

Variable	Variation explained (%)	df	SS	MS	Pseudo *F*	*P*
Invasion age	3.78	2	0.54	0.27	1.09	0.090
Soil ammonium	1.30	1	0.19	0.18	0.75	0.634
Soil nitrate	1.61	1	0.23	0.23	0.93	0.060
Soil phosphorus	1.43	1	0.20	0.20	0.83	0.380
Soil pH	3.81	1	0.54	0.54	2.21	0.075
Residuals	88.07	51	12.57	0.25		
Total	100	57	14.27			

Table 3.4 Coefficient estimates, *t*-statistics, and *P* values for each fixed effect contrast in generalised linear mixed models for AM fungi from roots of *V. rossicum* from 15 sites in three invasion ages: young (<12 years), intermediate (50-60 years), and old (100+ years). AM fungal species richness was a Poisson response. AM fungal abundance was a binomial response as the proportion of reads of AM fungi. Site was a random effect. Standard errors are in brackets.

Model	Invasion age	Coefficient (standard error)	*t*	*P*
AM fungal species richness	Intercept	2.99 (0.08)	37.63	0.000
	Contrast 1	-0.03 (0.06)	-0.55	0.589
	Contrast 2	-0.04 (0.10)	-0.42	0.685
AM fungal abundance	Intercept	-1.22 (0.14)	-8.53	0.000
	Contrast 1	-0.03 (0.10)	-0.32	0.756
	Contrast 2	-0.16 (0.17)	0.94	0.367

Note: Pre-planned contrasts: contrast 1: young compared to the mean of the intermediate and old invasion ages combined; contrast 2: intermediate compared to old invasion age.

Table 3.5 Results from permutational analysis of variance (PERMANOVA) testing potential predictors of AM fungal community composition from 454 pyrosequencing of 58 root samples of *V. rossicum* from 15 sites in three invasion ages: young (<12 years), intermediate (50-60 years), and old (100+ years).

Variable	Variation explained (%)	df	SS	MS	Pseudo *F*	*P*
Invasion age	3.33	2	0.83	0.42	0.94	0.472
Soil ammonium	2.18	1	0.54	0.54	1.23	0.207
Soil nitrate	2.06	1	0.52	0.52	1.16	0.620
Soil phosphorus	1.67	1	0.42	0.42	0.94	0.620
Soil pH	1.98	1	0.49	0.49	1.11	0.451
Residuals	88.78	50	22.23			
Total	100	56	25.04			

3.4.2. Plant-soil feedbacks along an invasion chronosequence

3.4.2.1 Overall feedback response

Vincetoxicum rossicum had higher total biomass and lower root-shoot ratios in live soil from all invasion ages compared to the sterile controls (Table 3.6, Fig. 3.5). This was true even for soil collected from uninvaded sites where *V. rossicum* had not trained the soil (Table 3.6, Fig. 3.5).

Table 3.6 Coefficient estimates, *t*-statistics, and *P* values for each fixed effect contrast in generalised linear mixed models for plant-soil feedback in terms of total plant biomass and root-shoot ratio of 133 plants of *V. rossicum* in soil from 20 sites in four invasion ages and sterile controls: uninvaded, young (<12 years), intermediate (50-60 years), and old (100+ years). Site was a random effect. Standard errors are in brackets.

Model	Contrast	Coefficient estimate (standard error)	*t*	*P*
Total plant biomass	Intercept	2.27 (0.15)	14.98	0.000
	Contrast 1	-0.33 (0.11)	-2.91	0.010
	Contrast 2	0.04 (0.07)	0.59	0.564
	Contrast 3	-0.17 (0.10)	-1.58	0.134
	Contrast 4	-0.03 (0.18)	-0.15	0.882
Root-shoot ratio	Intercept	2.33 (0.11)	22.02	0.000
	Contrast 1	0.22 (0.08)	2.81	0.013
	Contrast 2	-0.04 (0.05)	-0.69	0.501
	Contrast 3	0.05 (0.07)	0.67	0.515
	Contrast 4	-0.09 (0.13)	-0.68	0.508

Note: Pre-planned contrasts: contrast 1: sterile control compared to the mean of live soil combined (all invasion ages and uninvaded); contrast 2: uninvaded compared to the mean of young, intermediate, and old invasion ages combined; contrast 3: young compared to the mean of the intermediate and old invasion ages combined; contrast 4: intermediate compared to old invasion age.

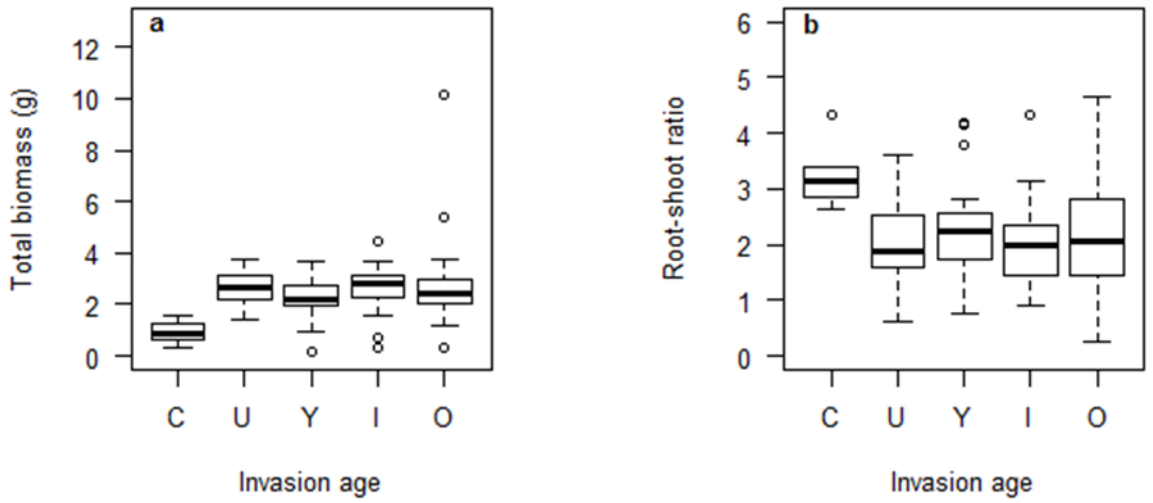

Fig. 3.5 Boxplots of plant-soil feedback responses of *V. rossicum* measured as (a) total dry biomass (g), and (b) root-shoot ratio from 133 plants from 20 sites of four invasion ages and sterile controls. C=sterile control, U=uninvaded, Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). The box represents the interquartile range, the band represents the median, and the whiskers indicate data within 1.5 times the interquartile range. Points are outliers beyond the whisker range. Statistically significant pre-planned contrasts are indicated by lines and an asterisk (*P <0.05, Table 3.6).

Table 3.7 Coefficient estimates, *t*-statistics, and *P* values for each fixed effect contrast in a generalised linear mixed model for plant-soil feedback with Poisson response for number of flowers and seed pods on 133 *V. rossicum* plants in soil from 20 sites in four invasion ages and sterile controls: uninvaded, young (<12 years), intermediate (50-60 years), and old (100+ years). Site was a random effect. Standard errors are in brackets.

Model	Contrast	Coefficient (standard error)	*t*	*P*
Number of flowers and/or seed pods	Intercept	0.82 (0.53)	1.54	0.128
	Contrast 1	-0.69 (0.52)	-1.32	0.204
	Contrast 2	0.06 (0.08)	0.80	0.436
	Contrast 3	-0.17 (0.12)	-1.39	0.182
	Contrast 4	0.20 (0.19)	1.05	0.309

Note: Pre-planned contrasts: contrast 1: sterile control compared to the mean of live soil combined (all invasion ages and uninvaded); contrast 2: uninvaded compared to the mean of young, intermediate, and old invasion ages combined; contrast 3: young compared to the mean of the intermediate and old invasion ages combined; contrast 4: intermediate compared to old invasion age.

3.4.2.2. Was negative feedback stronger over time?

Contrasting with my hypothesis, there was no change in total biomass or root-shoot ratio with increasing invasion age and no evidence for stronger negative feedback over invasion time (Table 3.6, Fig. 3.5). Of the 133 plants, 47 had seed pods: 13 in uninvaded, 11 in young, 13 in intermediate, and 10 in old invasion ages. Eighty had flowers: 21 in uninvaded, 17 in young, 25 in intermediate, 16 in old invasion ages, and one in the sterile soil. In terms of fitness, there was no relationship between invasion age and number of flowers and seed pods (Table 3.7, Fig. 3.6).

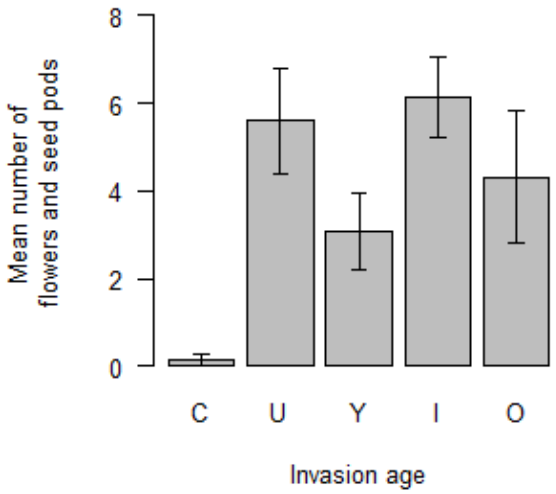

Fig. 3.6 Barplot of plant-soil feedback response of *V. rossicum* measured as the mean number of flowers and seed pods from 133 plants from 20 sites of four invasion ages and sterile controls. C=sterile control, U=uninvaded, Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). Error bars are standard errors of the mean.

3.4.2.4. Was there more pathogen damage in older invasion ages?

There was no increase in pathogen damage in terms of signs of decay or discolouration, or chew marks on plants growing in soil from sites of different invasion ages (Table 3.8, Fig. 3.7). There were no differences in the proportion of intersections with signs of decay in sterile controls compared to live soil (Table 3.8, Fig. 3.7).

3.4.2.3. Were there differences in colonisation by AM and non-AM fungi?

A high proportion of root intersections were colonised by AM fungi in all invasion ages, with an overall mean of 0.62 ± 0.02 intersections colonised (range: 0.21-0.91; Fig. 3.8). Roots from young invasion age had significantly lower AM fungal colonisation than the intermediate and old invasion ages (Table 3.9, Fig. 3.8). Roots in uninvaded soil had a significantly higher proportion of arbuscules than roots from all of the invasion ages (Table 3.9, Fig. 3.8). There were no

significant differences in vesicles or colonisation by non-AM fungi among invasion ages (Table 3.9, Fig. 3.8). Sterile controls showed no fungal colonisation.

Table 3.8 Coefficient estimates, *t*-statistics, and *P* values for each fixed effect contrast in generalised linear mixed models for binomial response of proportion of *V. rossicum* root intersections with signs of root decay, and signs of chew marks on 63 *V. rossicum* plants in soil from 20 sites in four invasion ages and sterile controls: sterile control, uninvaded, young (<12 years), intermediate (50-60 years), and old (100+ years). Site was a random effect. Standard errors are in brackets.

Model	Contrast	Coefficient (standard error)	*t*	*P*
Signs of root decay	Intercept	-2.28 (0.14)	-16.21	<0.001
	Contrast 1	-0.15 (0.12)	-1.25	0.229
	Contrast 2	-0.11 (0.06)	-1.76	0.098
	Contrast 3	0.00 (0.08)	0.00	0.999
	Contrast 4	0.09 (0.13)	0.67	0.512
Signs of chew marks	Intercept	-5.12 (0.28)	-18.41	<0.001
	Contrast 1	-0.14 (0.24)	-0.61	0.552
	Contrast 2	-0.04 (0.11)	-0.39	0.700
	Contrast 3	-0.09 (0.15)	-0.56	0.582
	Contrast 4	-0.38 (0.25)	-1.51	0.151

Note: Pre-planned contrasts: contrast 1: sterile control compared to the mean of live soil combined (all invasion ages and uninvaded); contrast 2: uninvaded compared to the mean of young, intermediate, and old invasion ages combined; contrast 3: young compared to the mean of the intermediate and old invasion ages combined; contrast 4: intermediate compared to old invasion age.

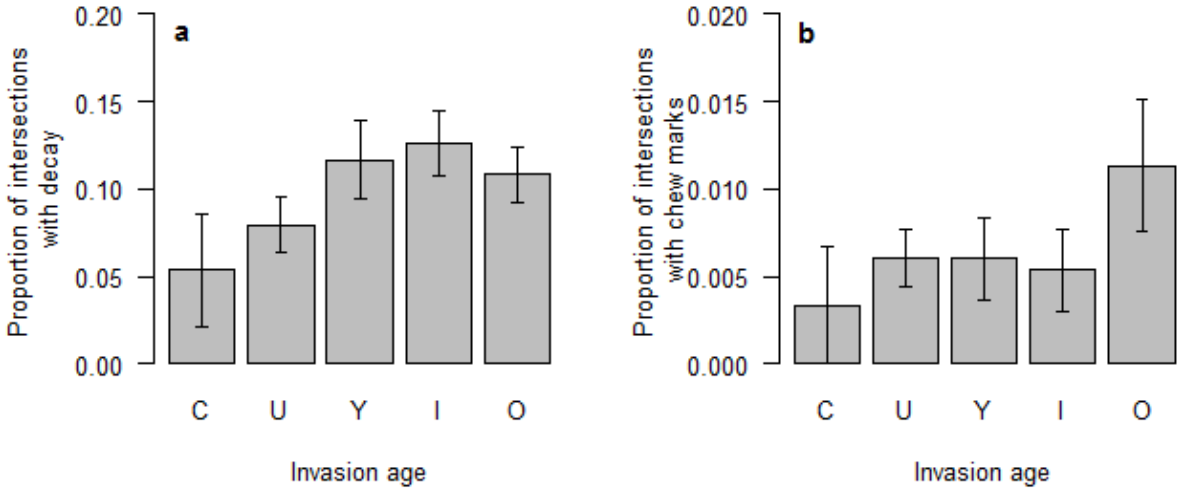

Fig. 3.7 Mean proportion of 100 root intersections of *V. rossicum* with a) signs of root decay or discoloration, and b) evidence for chew marks for 63 plants from 20 sites of four invasion ages and sterile controls. C=sterile controls, U=uninvaded, Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). Error bars are standard errors of the mean. Each bar has N=15.

Table 3.9 Coefficient estimates, *t*-statistics, and *P* values for each fixed effect contrast in generalised linear mixed models for binomial response of proportion of *V. rossicum* root intersections containing AM fungi, arbuscules, vesicles, and non-AM fungi on 60 *V. rossicum* plants in soil from 20 sites in four invasion ages: uninvaded, young (<12 years), intermediate (50-60 years), and old (100+ years). Site was a random effect. Standard errors are in brackets. Sterile controls showed no colonisation and were not included in these analyses.

Model	Contrast	Coefficient estimate (standard error)	*t*	*P*
AM fungi	Intercept	0.49 (0.09)	5.57	0.000
	Contrast 1	0.07 (0.05)	1.43	0.173
	Contrast 2	-0.19 (0.07)	-2.70	0.016
	Contrast 3	0.12 (0.13)	0.92	0.370
Arbuscules	Intercept	-2.33 (0.11)	-20.87	0.000
	Contrast 1	0.20 (0.06)	3.50	0.003
	Contrast 2	-0.20 (0.10)	-2.02	0.060
	Contrast 3	0.11 (0.15)	0.69	0.500
Vesicles	Intercept	-3.05 (0.23)	-13.39	0.000
	Contrast 1	0.14 (0.12)	1.13	0.277
	Contrast 2	-0.40 (0.21)	-1.94	0.071
	Contrast 3	0.24 (0.30)	0.81	0.428
Non-AM fungi	Intercept	-2.27 (0.10)	-22.04	0.000
	Contrast 1	0.01 (0.06)	0.15	0.881
	Contrast 2	-0.16 (0.09)	-1.85	0.083
	Contrast 3	-0.03 (0.14)	-0.22	0.830

Note: Pre-planned contrasts: contrast 1: uninvaded compared to the mean of young, intermediate, and old invasion ages combined; contrast 2: young compared to the mean of the intermediate and old invasion ages combined; contrast 3: intermediate compared to old invasion age.

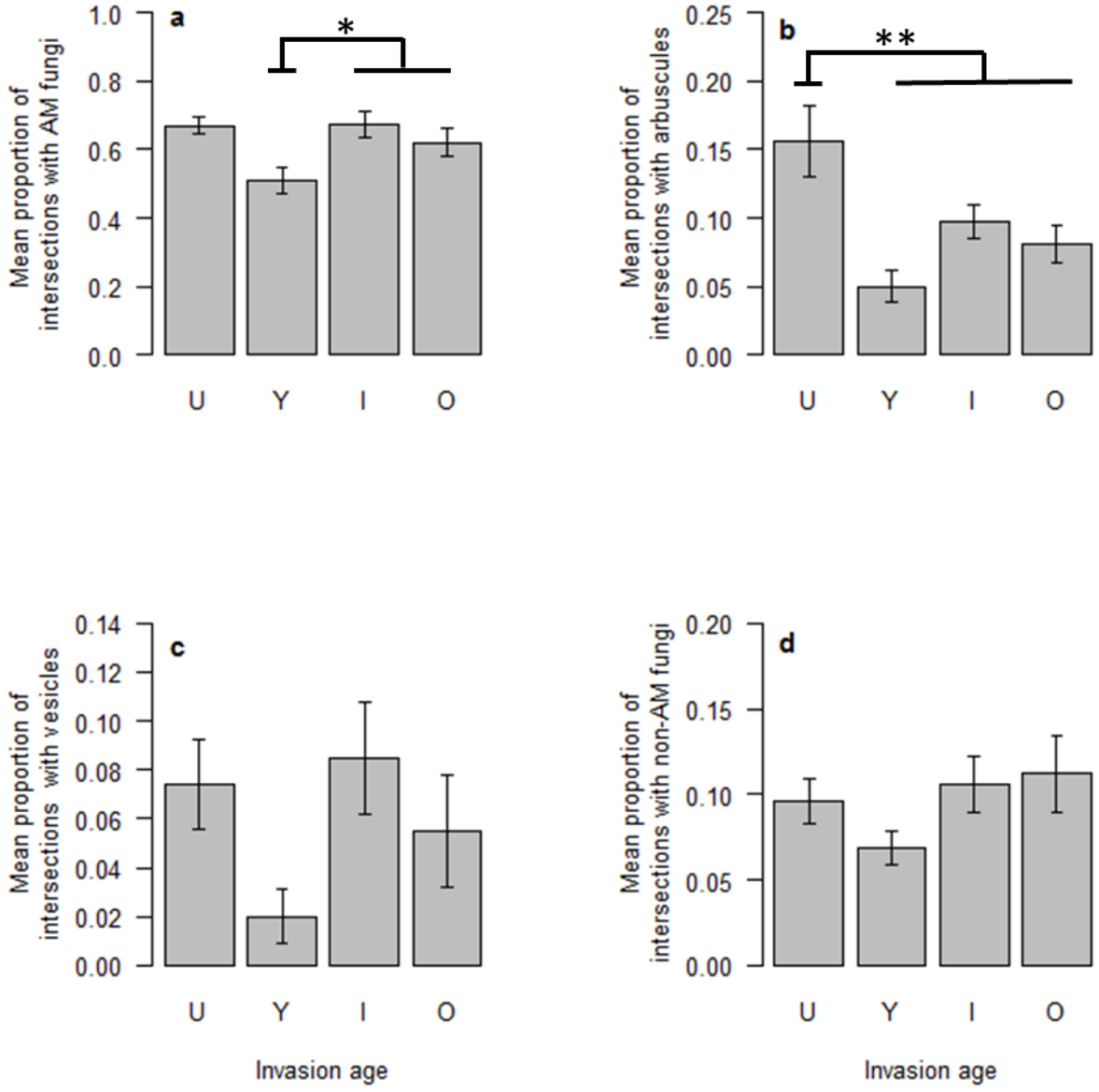

Fig. 3.8 Mean proportion of 100 root intersections of *V. rossicum* with (a) AM fungi, (b) arbuscules, (c) vesicles, and (d) non-AM fungi for 60 plants from 20 sites of four invasion ages. U=uninvaded, Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). Sterile controls showed no colonisation and are not shown. Error bars are standard errors of the mean. Statistically significant pre-planned contrasts are indicated by lines and asterisks (P* <0.05, ** *P* <0.01, Table 3.9).**

3.5. Discussion

This study combined high throughput sequencing data with a plant-soil feedback study along an invasion chronosequence and represents a unique way to assess long term changes in root-associated fungi and address questions surrounding pathogen accumulation. It was expected that *V. rossicum* would experience stronger negative feedback and pathogen damage in soils from sites of older invasion due to pathogen accumulation over 100 years. However, 454 pyrosequencing data on root-associated fungal communities from field roots did not support this and there was no evidence for accumulation of fungi that are known pathogens on other plant species in roots from older invasions (Table 3.2, Fig. 3.4). In addition, there were also no changes in AM or entire root-associated fungal species richness, abundance, or composition related to invasion age (Tables 3.1, 3.4, and 3.5; Figs. 3.2-3.4). Results from the plant-soil feedback experiment also did not support these hypotheses because negative feedback was not stronger in soils from older invasions (Figs. 3.5 and 3.6). The hypothesis for AM fungal colonisation was partially supported because colonisation by AM fungi was lower in soil from young invasions compared to intermediate and old invasions. However, AM fungal root colonisation in uninvaded soil was not different from that in soil from invaded sites (Table 3.9; Fig. 3.8). In combination, these results indicate that *V. rossicum* in southern Ontario does not conform to the PAID hypothesis (Flory and Clay, 2013). Instead, *V. rossicum* can associate with a wide range of soil fungi, and gains a net benefit from all soil biota.

3.5.1. Characterisation of root-associated fungal communities

This study contributes to the growing body of knowledge from high throughput sequencing data describing the microbial communities that reside within plants (Öpik et al., 2009; Bass and Richards, 2011; Dumbrell et al., 2011; Blaalid et al., 2012; Lekberg et al., 2012; Gaiero et al., 2013; Lindahl et al., 2013; Wehner et al., 2013; Ohsowski et al., 2014). A total of 478 fungal OTUs at 97% sequence similarity occurred in the 58 *V. rossicum* root samples and these represented a diversity of taxa. Most fungal taxa recorded here represent new records on this plant and only four of the 62 OTUs that were identified to genera have prior records on *V. rossicum*: *Colletotrichum* (Berner et al., 2011), *Fusarium*, *Glomus*, and *Plectosphaerella*

(Bongard et al., 2013). Genera with the most OTUs in the roots were in *Glomus*, *Rhizophagus*, and *Plectosphaerella* (Table S 3.4). Genera that were the most abundant in terms of reads were *Plectosphaerella*, *Glomus*, *Cyanonectria*, *Quadricrura*, and *Rhizophagus* (Table S 3.4). The abundance of *Glomus* and *Rhizophagus* supports previous evidence that *V. rossicum* is highly mycorrhizal (Smith et al., 2008; Bongard and Fulthorpe, 2013; this thesis chapter 2). In addition, these genera are both in the Glomeraceae family, which is thought to be better at protecting plants from pathogens compared to other families within the Glomeromycota (Maherali and Klironomos, 2007; Sikes et al., 2009), and may have neutralised the effects of the many fungi that are known pathogens on other plant species that were detected.

These data suggest that *V. rossicum* is able to associate with a broad range of fungal taxa. Other studies have indicated that plant host is an important driver of root-associated fungal community composition (Broz et al., 2007; Kardol et al., 2007; Hausmann and Hawkes, 2010; Pendergast et al., 2013; Wehner et al., 2013) and that this may take decades to become evident (Blaalid et al., 2012; Wu et al., 2013). However, in this study these data showed no significant relationship between invasion age and root-associated fungal community composition (Table 3.1, Fig. 3.3), although some Orders were more abundant in particular invasion ages than others (Fig. 3.2). For example, there was a higher proportion of Glomerales reads for the old invasion age, and these may have contributed to pathogen-protection (Maherali and Klironomos, 2007; Sikes et al., 2009). The non-significance of invasion age for root-associated fungal composition may have been overridden by differences in plant age, which has been shown to be important, at least for AM fungi (e.g., Schreiner and Mihara, 2009; Hart et al., 2014). Although care was taken to isolate fungal DNA from roots from crowns of similar size, it is possible that individual plants were not the same age. Surrounding plant species composition and localised environmental factors are also known to be important determinants of root and soil fungi (Hausmann and Hawkes, 2009; Blaalid et al., 2014; Peay and Bruns, 2014). This study was not designed to explicitly test the relative importance of these factors, but the lack of clustering of root samples by site (Fig. 3.3) indicates that these may not be important for root-associated fungal composition on *V. rossicum*. However, similar to this study, others have also found significant within-site variation in root fungi (Blaalid et al., 2014; Peay and Bruns, 2014). More work combining controlled experiments with high throughput sequencing of soil, as well as roots of surrounding plant species, would help elucidate if *V. rossicum* is a fungal generalist and if it is an important trait for explaining invasion success.

It was further surprising that none of the soil parameters were significant predictors of root-associated fungal community composition. These soil parameters are known to be important determinants of root-associated fungal community composition in other plant species and the inability to detect a pattern here suggests that *V. rossicum* is a generalist that is able to form associations with a large group of soil fungi in diverse soils. In particular, a wide range in soil pH was represented in the soils collected and is known to be important for root-associated and soil fungal communities (Stotzky, 1997; Lekberg et al., 2007). It was expected that soil phosphorus would be a significant predictor of root-associated, and particularly AM, fungal composition, because some groups of fungi are known to be better at taking up and transferring phosphorus to the plant compared to others, and therefore may be selected for depending on phosphorus availability (Jakobsen et al., 1992; Dickson et al., 1999; Kiers et al., 2011). For example, species within Gigasporaceae are better at phosphorus transfer than those in Glomeraceae (Maherali and Klironomos, 2007), but no genera within this family were detected in the roots (Table S 3.4). It is possible that *V. rossicum* does not form associations with this family. It is also possible that the surface disinfection may have eliminated Gigasporaceae DNA from the samples because it has high hyphal mass on the root exterior (Hart and Reader, 2002), or it could be due to a primer bias. However, work with other primers and rDNA regions with non-surface disinfected roots of *V. rossicum* have not detected Gigasporaceae either (Bongard et al. 2013; this thesis chapter 2).

Well-established fungal-specific primers were used for the 454 pyrosequencing and did not detect non-fungal sequences. The greater representation of Ascomycota compared to Basidiomycota in the 454 data could be because Ascomycota is the largest fungal Phylum (Kirk et al., 2001), or because the ITS region is smaller in Ascomycota and may have led to preferential amplification of this group (Bellemain et al., 2010). The use of abundance data in terms of number of sequences from 454 pyrosequencing is debated (Amend et al., 2010), and using nested PCR can exacerbate this (Lindahl et al., 2013). This is especially controversial for ITS sequences because fungi contain multiple copies of this region. Importantly, results were unchanged whether using abundance data or presence-absence data in terms of number of OTUs (Tables 3.2 and 3.4). Clustering fungal ITS sequences at 97% similarity is considered appropriate in most cases but incorrect clustering for some species is recognised (Nilsson et al., 2008; Kunin et al., 2010; Lekberg et al., 2013; Blaalid et al., 2013; Powell and Sikes, 2014). While it is possible that clustering at a different level of similarity could have led to different

conclusions in this dataset, studies investigating different cut-off levels have indicated that key ecological patterns are detectable at 97% (Blaalid et al., 2013; Lekberg et al., 2014). In addition, the use of the UNITE database to assign taxonomic identities, where reference sequence identities and names are curated by researchers with taxonomic knowledge and compared to the literature, provides high confidence in the identities obtained (Kõljalg et al., 2013).

Many fungi that are known pathogens on other plant species were detected within the roots but there was no pattern of accumulation in relation to invasion age over 100 years. It is possible that *V. rossicum* will not accumulate pathogens and result in predictable declines (Flory and Clay, 2013). Alternatively, pathogen accumulation could become evident as *V. rossicum* is established for longer. For example, Hawkes (2007) suggested that 150-200 years is required for pathogen load in exotic species to match that of native species. One particular pathogen may have a more detrimental effect on an individual plant or population than pathogen load *per se*, and this can form the basis of biological control. At one site in New York State, where *V. rossicum* is also invasive, *Sclerotium rolfsii* has been observed to cause wilting and mortality of *V. rossicum* (Gibson, 2012; Gibson et al., 2014). Interestingly, *V. rossicum* was not observed at that site until after 1995 (Milbrath, L. R., and Southby, C., pers. comm). There have been no observations of this pathogen in Ontario and it was not detected in the DNA sequences obtained from the field roots (Tables S 3.2-S 3.4). However, pathogens require time to reach an appropriate density to disperse to the levels and over the distances required for an epidemic to occur (Gilbert and Parker, 2006; Kivlin et al., 2014). Therefore, once *S. rolfsii* or another successful pathogen emerges and propagules increase, *V. rossicum* populations could decline. For example, at a range of sites in the USA invaded by *Microstegium vimineum*, Flory et al. (2011) found five strains of a leaf lesion-causing fungus, *Bipolaris* sp., that reduced biomass and reproduction of the invasive grass. It is possible that the criteria used for defining pathogens was too broad, so further work is required to test the effects of pathogens that associate with *V. rossicum.*

This study supports growing evidence suggesting that *V. rossicum* is able to invade many different soil types and associate with a broad range of fungal biota, which may contribute to its invasion success (Pringle et al., 2009; Bongard et al., 2013; Sanderson et al., 2015; this thesis chapter 2). *Vincetoxicum rossicum* produces a secondary compound that appears to be unique to North America, (-)-antofine, which has been shown to have antifungal properties

(Cappuccino and Arnason, 2006; Mogg et al., 2008). It has been suggested that (-)-antofine is a novel weapon in North America, where the invading plant may gain a competitive advantage by possessing unique chemicals to which native plants are not pre-adapted (Callaway and Ridenour, 2004; Callaway et al., 2005; Cappuccino and Arnason, 2006; Mogg et al., 2008; Lankau et al., 2009). However, the present study and others have demonstrated that *V. rossicum* associates with a diversity of taxa across the fungal kingdom (Greipsson and DiTommaso, 2006; Smith et al., 2008; Berner et al., 2011; Weed et al., 2011; Gibson, 2012; Bongard et al., 2013), which does not support the hypothesis that (-)-antofine is an important mechanism of invasion for *V. rossicum*.

3.5.2. Plant-soil feedbacks along an invasion chronosequence

Vincetoxicum rossicum appears to experience a net benefit from soil biota and enhanced mutualisms in its invasive range. In contrast with other plant-soil feedback studies incorporating invasion time (Diez et al., 2010; Dostál et al., 2013), there was no evidence for stronger negative plant-soil feedback in soil from older invasion ages of *V. rossicum* (Tables 3.6 and 3.7; Figs. 3.5 and 3.6). Interestingly, although significant differences in biomass and fitness were not observed, there were significant differences in colonisation by AM fungi (Table 3.9, Fig. 3.8). Plants in uninvaded soil had significantly more arbuscules than all invasion ages (Table 3.9, Fig. 3.8). This shows that *V. rossicum* can rapidly form associations with AM fungi when it first invades and also gain access to soil nutrients from the fungi via arbuscules (Smith and Read, 2008). *Vincetoxicum rossicum* also had lower AM fungal colonisation in young invasion age, but again, this was not reflected in differences in biomass and fitness (Tables 3.6, 3.7 and 3.9, Figs. 3.5, 3.6 and 3.8). Results indicate that *V. rossicum* generally benefits from soil biota such as AM fungi regardless of invasion age, and that other soil biota may compensate for low AM fungal colonisation.

Pathogens can evolve rapidly to be able to infect a host, but this may not be reflected in increased damage or disease (Hawkes, 2007; Gilbert and Parker, 2010). Although root decay, discolouration,n and non-AM fungal colonisation were observed in the plant-soil feedback experiment, these did not differ from controls (Fig. 3.7). There are a range of reasons why the

many potential pathogens detected using 454 sequencing did not cause disease symptoms in *V. rossicum*. It is possible that negative feedbacks would have been observed under different environmental conditions, in plants of different ages, or different plant genotypes. Alternatively, a pathogen species able to infect *V. rossicum* may have been present but not a host specific strain or in the appropriate stage of the life cycle (Agrios, 2005). Importantly, anamorph fungal forms (asexual) are more likely to cause disease symptoms than teleomorph fungal forms (sexual; Agrios, 2005). Up until recently, these morphs have been given different names, because they are morphologically distinct (Alexopoulos et al., 1996; Agrios, 2005; Domsch et al., 2007; Hawksworth, 2011). The increasing use of sequences instead of morphological data has led to one name for both morphs (i.e., the holomorph), but sequences do not allow us to distinguish which morph was present in the sample (Shenoy et al., 2007; Hawksworth, 2011). These difficulties highlight the need to combine high throughput sequence data with traditional plant pathogenicity testing to be able to assess function (van der Putten et al., 2007a).

Multiple invasive species have been shown to have higher biomass when grown in live soil from their invaded range compared to sterile soil, while simultaneously showing the opposite response to soil from their native range (Callaway et al., 2004; Nijjer et al., 2007; Gundale et al., 2014; Maron et al., 2014). A meta-analysis by Kulmatiski et al. (2008) showed that even when invasive plants show negative feedbacks, it is often less negative compared to native and non-invasive exotic plants. This indicates that indirect interactions between invasive plants and surrounding plants via soil biota may be an important mechanism for dominance (Eppinga et al., 2006). Feedbacks can also change over time. For example, Hawkes et al. (2013) measured feedback at regular intervals for 19 months and showed that the direction of feedback changed over time, and chapter 2 of this thesis shows that fine scale temporal changes in root-shoot ratios can differ in previously uninvaded and invaded soils over 29 weeks. It is possible that different results may have been obtained if the plants had been grown for a different period of time. However 12 weeks is a commonly used time for a feedback study. In addition, the results from the sequences and the plant-soil feedback experiments were consistent, in terms of no pathogen accumulation or growth differences with invasion age.

Combining the results from 454 data of field roots and a plant-soil feedback study showed that *V. rossicum* associated with many fungi that are known pathogens on other plant species, but that ~100 years may not have been enough time for the plant to have accumulated

a pathogen load capable of causing significant declines in biomass or fitness. These results contrast with the hypothesis that invasive species accumulate parasites and pathogens over time (Hawkes, 2007; Mitchell et al., 2010; Flory et al., 2011). However, Hawkes (2007) points out that accumulation of pathogens and parasites may not necessarily lead to declines in fitness or invasion ability. It is possible that effects of the large number of pathogens on *V. rossicum* were offset by pathogen-protecting AM fungi in the Glomeraceae family (Newsham et al., 1995; Sikes et al., 2009; Wehner et al., 2010; Lewandowski et al., 2013). It is also possible that the classification of pathogens was too broad. However, the plant-soil feedback experiment also suggests that there were no effects of the different root-associated fungal communities on plant growth and that pathogen accumulation has not occurred over time. Taken together, these results suggest that *V. rossicum* can associate with many fungi at multiple locations to gain a net benefit, and this may be a mechanism underlying its invasion success.

3.6. Conclusions

Plant invasion success is difficult to predict and associations with soil microbial communities may be more important for some plant species than others. This study highlights the need to combine studies that integrate both the identification of potential pathogens and functional responses to those pathogens, because high pathogen loads may not be reflected in reduced plant growth. These data indicate that invasion success of *V. rossicum* may at least in part be explained by its ability to associate with many root-associated fungal taxa and gain a net benefit from all soil biota in its invasive range. It is possible that pathogen accumulation will become more important as *V. rossicum* is established for longer; data from Hawkes (2007) and Diez et al. (2010) suggest that approximately 200 years may be required. However, Flory et al. (2011) and Dostál et al. (2013) showed pathogen accumulation and stronger negative feedback leading to significant declines after just 100 and 50 years of invasion respectively. In contrast, my results for *V. rossicum* do not support the PAID hypothesis (Flory and Clay, 2013) and indicate that management is still required for population control. Future work combining plant-soil feedback studies with sequencing of root-associated or soil biota will help to elucidate the generality of the PAID hypothesis in relation to soil communities.

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3.7. Supplementary material

Table S 3.1 Site names, invasion age category, estimated year of invasion, and the source for the invasion age for the 20 sites where soil was collected and the 15 invaded sites where plant samples were also collected.

Site name	Invasion age category	Estimated year of invasion	Source for age estimate
Charles Sauriol Reserve, Don Valley, Toronto	Old	~1900	Scott 1913[1]
Rouge Park, Don Valley, Toronto	Old	~1900	Scott 1913[1]
Rouge Park Woodlands Area, Don Valley, Toronto	Old	~1900	Scott 1913[1]
Wexford Park, Don Valley, Toronto	Old	1920s	Royal Ontario Museum herbarium
Sunnybrook Park, Don Valley, Toronto	Old	1939	Royal Ontario Museum herbarium
Ottawa	Intermediate	1950	Marie-Victorin herbarium
Metro Tract, Georgina	Intermediate	1963	Royal Ontario Museum herbarium
Carleton University, Ottawa	Intermediate	1967	Marie-Victorin herbarium
Hamilton Royal Botanic Gardens	Intermediate	1969	Royal Botanic Gardens herbarium
Proctor Park, Brighton	Intermediate	1969	Marie-Victorin herbarium
Omemee	Young	2000s	Kawartha Lakes Conservation Authority
Juniper Trail, Kirkfield	Young	2003	Couchiching Conservancy
Brantford	Young	2008	Grand River Conservation Authority
Trent University, Peterborough	Young	2007-2012	Ministry of Natural Resources
Luther Marsh Wildlife Management Area	Young	~2012	Grand River Conservation Authority
Ken Reid Conservation Area, Kawartha Lakes	Uninvaded	N/A	Kawartha Conservation Authority
Starkey Hill, Guelph	Uninvaded	N/A	Grand River Conservation Authority
Porritt Tract, Aurora	Uninvaded	N/A	Regional Municipality of York
Seymour Conservation Area, Cambellford	Uninvaded	N/A	Lower Trent Conservation Authority
Juniper Trail, Kirkfield	Uninvaded	N/A	Couchiching Conservancy

[1]Scott, P.M. 1913. in: Faull, J.H. (Ed.), The Natural History of the Toronto Region. The Canadian Institute, Toronto, pp. 100–140.

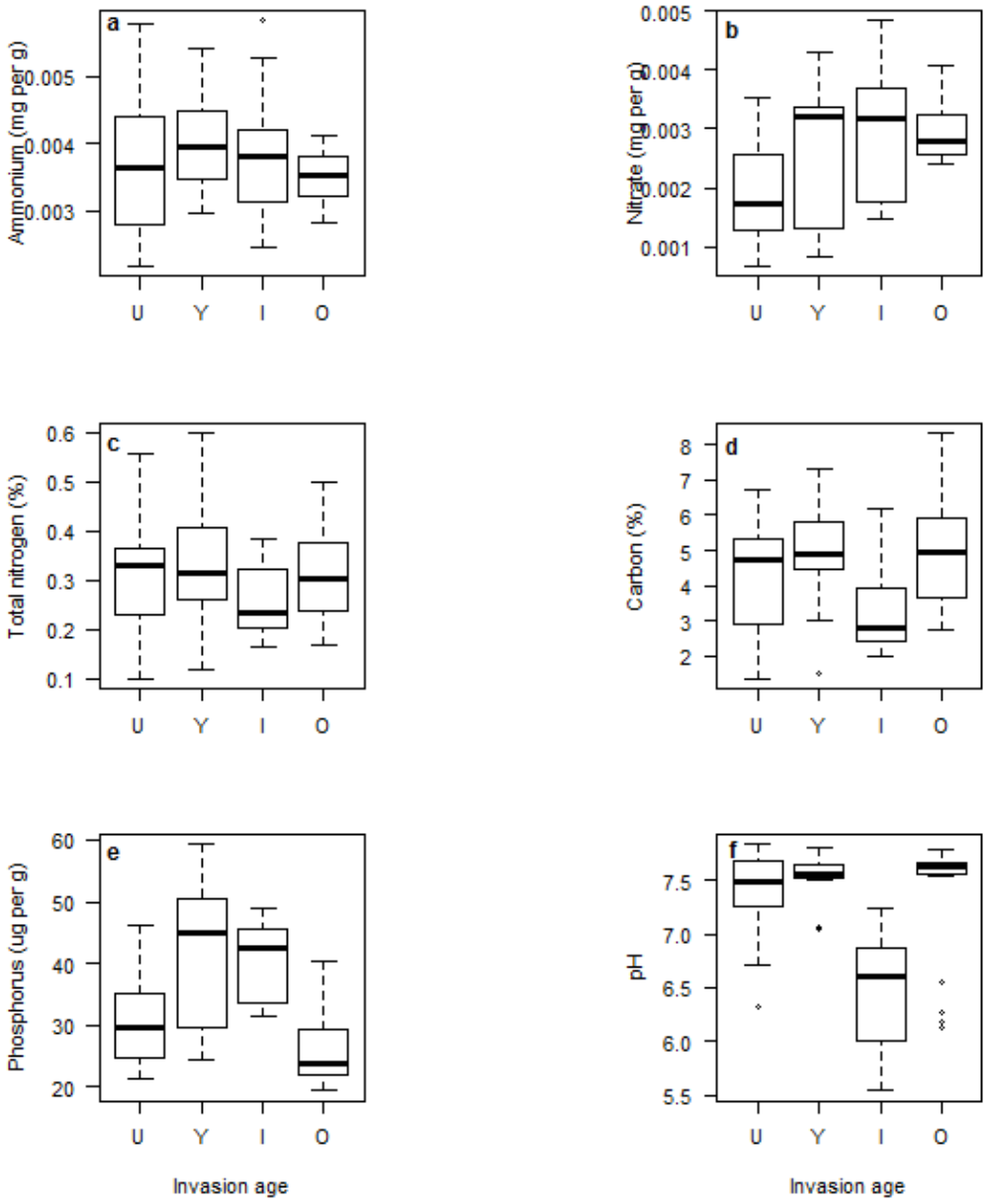

Fig. S 3.1 Boxplots of soil parameters from four soil samples from 20 sites showing a) ammonium (mg per g), b) nitrate (mg per g), c) total nitrogen (%), d) total carbon (%), e) phosphorus (µg per g), and f) pH, according to *V. rossicum* invasion age. U=uninvaded, Y=young invasion (<12 years), I=intermediate invasion (50-60 years), and O=old invasion (100+ years). The box represents the interquartile range, the band represents the median, and the whiskers indicate data within 1.5 times the interquartile range. Points are outliers beyond the whisker range.

Table S 3.2 Distribution of 478 OTUs across Orders from 454 pyrosequencing of 58 roots roots of *V. rossicum* from three invasion ages: young (<12 years), intermediate (50-60 years), and old invasion (100+ years). Data is based on the ITS region for fungi and RDP classification with confidence >0.5 (see Methods).

Order	Number of OTUs	Number of reads
Agaricales	4	2465
Cantharellales	1	71
Capnodiales	1	18
Chaetosphaeriales	2	2293
Chaetothyriales	2	2552
Coniochaetales	1	37
Diaporthales	4	328
Diversisporales	7	1411
Eurotiales	7	6086
Glomerales	221	95588
Helotiales	35	70927
Hypocreales	35	56440
Incertae sedis	39	87496
Lulworthiales	2	756
Mucorales	5	5113
Paraglomerales	5	1642
Pezizales	2	4820
Pleosporales	27	45695
Rhytismatales	3	1498
Russulales	2	139
Sordariales	1	150
Ustilaginales	1	150
Xylariales	3	689
not assigned at >0.5 confidence	68	20320
Total	478	406684

Table S 3.3 Distribution of 478 OTUs across Families from 454 pyrosequencing of 58 roots roots of *V. rossicum* from three invasion ages: young (<12 years), intermediate (50-60 years), and old invasion (100+ years). Data is based on the ITS region for fungi and RDP classification with confidence >0.5 (see Methods).

Family	Number of OTUs	Number of reads
Amphisphaeriaceae	1	3
Annulatascaceae	1	134
Anthracoideaceae	1	150
Bionectriaceae	3	6276
Ceratobasidiaceae	1	71
Chaetomiaceae	1	150
Chaetosphaeriaceae	2	2293
Chaetothyriaceae	1	2077
Claroideoglomeraceae	5	629
Coniochaetaceae	1	37
Davidiellaceae	1	18
Dermateaceae	2	1545
Diaporthaceae	4	328
Diatrypaceae	1	30
Diversisporaceae	3	1098
Gigasporaceae	3	291
Glomeraceae	216	94959
Glomerellaceae	5	2121
Helotiaceae	11	40619
Herpotrichiellaceae	1	475
Hyaloscyphaceae	12	22083
Lentitheciaceae	1	619
Leptosphaeriaceae	2	781
Lulworthiaceae	2	756
Magnaporthaceae	1	4
Montagnulaceae	1	2584
Myxotrichaceae	1	1408
Nectriaceae	32	50164
Niaceae	1	10
Paraglomeraceae	5	1642
Phacidiaceae	1	690
Phaeosphaeriaceae	5	5123
Phycomycetaceae	4	5108
Plectosphaerellaceae	31	83829
Pleomassariaceae	1	261

Table S 3.3 continued

Family	Number of OTUs	Number of reads
Pleosporaceae	3	11003
Pyronemataceae	2	4820
Rhytismataceae	3	1498
Russulaceae	1	100
Sporormiaceae	1	516
Strophariaceae	1	1427
Tetraplosphaeriaceae	7	22013
Trichocomaceae	7	6086
Typhulaceae	1	13
Vibrisseaceae	2	4360
not assigned at >0.5 confidence	86	26482
Total	478	406684

Table S 3.4 Distribution of 478 OTUs across Genera from 454 pyrosequencing of 58 roots roots of *V. rossicum* from three invasion ages: young (<12 years), intermediate (50-60 years), and old invasion (100+ years). Data is based on the ITS region for fungi and RDP classification with confidence >0.5 (see Methods). Genera that were classified as pathogens or mutualistic arbuscular mycorrhizal fungi (AMF) for the analyses are shown. An example reference for determining function is provided but are not exhaustive.

Genus	Number of OTUs	Number of reads	Pathogen or AMF	Example
Acephala	2	4360		
Acicuseptoria	1	273	pathogen	Quaedvlieg et al., 2013
Agrocybe	1	1427		
Alternaria	1	3435	pathogen	Couture et al., 2003
Aquaticola	1	134		
Cetraspora	3	291	AMF	www.mycobank.org
Chloridium	1	47		
Cistella	2	627	pathogen	Suto, 1997
Cladosporium	1	18	pathogen	Couture et al., 2003
Claroideoglomus	5	629	AMF	
Clonostachys	2	5852	pathogen	Zazzerini and Quaglia, 2010
Colletotrichum	5	2121	pathogen	Couture et al., 2003
Cryptosporiopsis	1	1540	pathogen	Couture et al., 2003
Cyanonectria	10	43474		
Cylindrium	1	157		
Cyphellophora	1	2077		
Diaporthe	3	287	pathogen	Couture et al., 2003
Epicoccum	2	7568	pathogen	Couture et al., 2003
Exophiala	1	475		
Funneliformis	1	31	AMF	
Fusarium	2	2536	pathogen	Couture et al., 2003
Gaeumannomyces	1	4	pathogen	Couture et al., 2003
Geejayessia	1	677	pathogen	Schroers et al., 2011
Gibellulopsis	2	6	pathogen	Kawaradani et al., 2013
Glomus	151	68976	AMF	
Haematonectria	2	525	pathogen	Hirooka et al., 2007
Humicola	1	150		
Hyaloscypha	2	2467		
Hydropisphaera	1	424	pathogen	James et al., 2006

Table S 3.4 continued.

Genus	Number of OTUs	Number of reads	Pathogen or AMF	Example
Lachnella	1	10	pathogen	Nakasone and Gilbertson, 1978
Lecythophora	1	37		
Leptosphaeria	1	508	pathogen	Couture et al., 2003
Lulworthia	1	261		
Mycoarthris	1	133		
Neonectria	1	59	pathogen	Castlebury et al., 2006
Oidiodendron	1	1408	pathogen	Couture et al., 2003
Paraglomus	5	1642	AMF	
Paraphaeosphaeria	1	2584	pathogen	Lee et al., 2005
Parastagonospora	5	5123	pathogen	Quaedvlieg et al., 2013
Paurocotylis	1	4723		
Penicillium	6	5930	pathogen	Couture et al., 2003
Phacidium	1	690	pathogen	Couture et al., 2003
Phaeomollisia	1	5		
Phomopsis	1	41	pathogen	Couture et al., 2003
Phycomyces	3	5103		
Plectosphaerella	28	83774	pathogen	Usami et al., 2012
Preussia	1	516		
Prosthemium	1	261	pathogen	Couture et al., 2003
Quadricrura	7	22013		
Redeckera	2	1062	AMF	
Rhizophagus	53	19833	AMF	
Rhizoscyphus	1	5		
Rhytisma	3	1498	pathogen	Hou and Piepenbring, 2005
Russula	1	100		
Schizonella	1	150	pathogen	Deml et al., 1980
Septoglomus	6	322	AMF	
Setoseptoria	1	619	pathogen	Quaedvlieg et al., 2013
Talaromyces	1	156		
Thanatephorus	1	71	pathogen	Couture et al., 2003
Tricladium	3	346		
Typhula	1	13	pathogen	Couture et al., 2003
Volutella	1	276	pathogen	Bai et al., 2012
not assigned at >0.5 confidence	127	96824		
Total	478	406684		

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Usami, T., et al. 2012. J. Gen. Plant Pathol. 78: 368–371.

Zazzerini, A., and Quaglia, M. 2010. Dis. Notes 94: 372.

Chapter 4. Contrasting effects of known fungal pathogens isolated from *Vincetoxicum rossicum* on its host and co-occurring native plant species

4.1. Abstract

Invasive plants can accumulate pathogens that reduce their growth, but these pathogens may be relatively more detrimental to growth of surrounding native plants. In these situations, the invasive plant acts as a pathogen reservoir, which could lead to apparent competition between invasive and native plants and alter the structure of plant communities. In this study, root-associated fungi were isolated from a highly invasive plant, *Vincetoxicum rossicum* (Apocynaceae). The growth effects of some of these fungi were then tested on *V. rossicum* and two co-occurring native plants, *Asclepias syriaca* (Apocynaceae) and *Solidago canadensis* (Asteraceae). I hypothesised that *V. rossicum* would not experience pathogenic effects from the tested fungi. Since host-switching is more likely between closely related species, I hypothesised that the tested fungi would be more pathogenic on *A. syriaca*, which is more closely related to *V. rossicum* than *S. canadensis*. A total of 18 taxa of root-associated fungi were isolated from *V. rossicum,* 15 of which have been previously reported as pathogens. Seven fungal isolates were used in a growth chamber experiment to test if they could re-colonise and cause growth effects on *V. rossicum*, and three of these were also tested on *A. syriaca* and *S. canadensis*. Contrasting with my hypothesis, all of the seven tested fungi increased total biomass and/or root-shoot ratio of *V. rossicum* compared to uninoculated controls. However, a mix of three fungi, *Cadophora* sp., *Ilyonectria radicicola*, and *Macrophomina phaseolina*, reduced the total biomass of *S. canadensis*, whereas no effect was observed for each individual fungal isolate alone. Inoculation with any fungi did not affect the growth of *A. syriaca*. These results indicate that the invasive *V. rossicum* gained benefit from multiple fungal taxa that can reduce growth of

a co-occurring native plant, *S. canadensis*. This study further contributes to knowledge of specific plant-fungal interactions in natural communities.

Keywords: pathogen reservoir, spillover, invasion, *Vincetoxicum rossicum*, *Asclepias syriaca*, *Solidago canadensis*

4.2. Introduction

One mechanism that has been suggested to explain the success of exotic invasive species is the enemy release hypothesis, in which restrictions on growth and fitness are not limited by co-evolved parasites and pathogens that are left behind when they invade a new range (Keane and Crawley, 2002; Shea and Chesson, 2002; Colautti et al., 2004). However, over time, invasive plants may accumulate pathogens in their introduced range (Mitchell and Power, 2003; Flory et al., 2011). If pathogenic agents are identified on invasive plants, bioaugmentation could reduce the lengthy process of screening potential biological control agents (e.g., Epstein et al., 1997). Multiple studies have compared pathogen or enemy loads on invasive plants in their native and invasive ranges, or with those of natives in the invasive range (Mitchell and Power, 2003; Torchin and Mitchell, 2004; Agrawal et al., 2005; Hawkes, 2007). However, surprisingly few studies have included tests of how isolated pathogens impact plant fitness (e.g., Mills and Bever, 1998; Flory et al., 2011; Li et al., 2014).

Due to host specificity and differential growth responses, it is unclear how pathogen accumulation on invasive plants may impact surrounding native plant species and subsequently shape plant community structure (Dobson and Crawley, 1994; Mangla et al., 2008; Beckstead et al., 2010; Flory et al., 2011; Mordecai, 2011; Li et al., 2014). Invasive plants could associate with microbes that are less pathogenic to themselves than they are to co-occurring native species (Eppinga et al., 2006). A relative reduction in growth of co-occurring plants due to the host-specific pathogens could enable the invasive plant to increase in abundance in the plant community (Eppinga et al., 2006). In these situations invasive plants can be considered pathogen “reservoirs”, where spillover of the accumulated pathogens impacts native plants and could lead to pathogen-mediated apparent competition between invasive and native plants

(Daszak et al., 2000; Power and Mitchell, 2004; van der Putten et al., 2007a; Mangla et al., 2008; Kelly et al., 2009; Beckstead et al., 2010; Flory and Clay, 2013; Li et al., 2014). Pathogen-mediated apparent competition is where the reduction in growth of a plant species appears to be due to direct competition for shared resources, but is actually indirect because it is the pathogen that is reducing fitness (Daszak et al., 2000; Malmstrom et al., 2005; Beckstead et al., 2010). For example, Mangla et al. (2008) showed that the invasive *Chromolaena odorata* increased the abundance of spores of *Fusarium semitectum* in the soil, which were shown to reduce the growth of two native plants.

Vincetoxicum rossicum (Kleopow) Barbar. (Apocynaceae; syn*. Cynanchum rossicum* (Kleopow) Borhidi; dog-strangling vine) is a highly invasive plant in parts of North America, including southern Ontario, Canada (Sheeley and Raynal, 1996; Cappuccino et al., 2002). It becomes dominant and can compete with surrounding vegetation (Cappuccino, 2004; DiTommaso et al., 2005a; Averill et al., 2011; Anderson, 2012). *Vincetoxicum rossicum*'s ability to establish under a range of light, moisture, and climate conditions, as well as in many different soil and vegetation types (DiTommaso et al., 2005a; Averill et al., 2011; Sanderson and Antunes, 2013; Sanderson et al., 2015), mean that its invasion could impact a wide range of native plant species, particularly if it acts as a pathogen reservoir. Two native species that often co-occur with *V. rossicum* are *Asclepias syriaca* L. (Apocynaceae) and *Solidago canadensis* L. (Asteraceae; Agrawal and Kotanen, 2003; Cappuccino, 2004; Averill et al., 2010; Kricsfalusy and Miller, 2010; Bongard et al., 2013). Pathogens may be more likely to spillover from *V. rossicum* to *A. syriaca* because they are closely related (Daszak et al., 2000; Keane and Crawley, 2002; Colautti et al., 2004; Torchin and Mitchell, 2004; Gilbert and Parker, 2006). *Vincetoxicum rossicum* is also competitive against *S. canadensis* and can reduce its biomass (Sanderson and Antunes, 2013). This reduction in biomass could be due to apparent competition that is mediated by pathogens.

The objective of this study was to investigate the hypothesis that *V. rossicum* associates with root fungi that reduce the growth of surrounding native plants more than itself, which may be a mechanism to explain its invasion success and dominance in southern Ontario. First, fungi were isolated and identified from surface disinfected roots of *V. rossicum* plants. Three of these fungi were subsequently used to conduct pathogenicity tests on *V. rossicum* and on *A. syriaca* and *S. canadensis*. Previous data using DNA fingerprinting and high throughput sequencing has

shown that *V. rossicum* harbours many fungi that are known pathogens on other plant species in its roots, but that these may not negatively impact its growth (Bongard and Fulthorpe, 2013; Bongard et al., 2013; this thesis chapter 3). Therefore, I expected to isolate multiple fungal isolates that are known pathogens on other plant species from *V. rossicum* that would not be pathogenic on *V. rossicum*. However, I expected that these fungi would be pathogenic on native plants, particularly on *A. syriaca* because it is in the same family as *V. rossicum*.

4.3. Methods

4.3.1. Isolation and identification of fungi from roots

4.3.1.1. Sample preparation

To gain a sample of fungi that associate with the roots of *V. rossicum* in its invasive range, four plants were collected from each of eight sites in southern Ontario (32 plants total). Plants were collected within 5 m of each other and there were no observations of diseased plants at any site. Whole plants, including roots and surrounding soil, were collected with a spade and placed into separate plastic bags. Plants were transported on ice, stored at 4˚C, and processed within four days of collection. *Vincetoxicum rossicum* forms dense root masses with intertwined roots stemming from multiple crowns, so subsamples of roots were collected from one crown of each plant. Roots were washed thoroughly in tap water, dried with a paper towel, and cut into lengths of approximately 1 cm. Root pieces were surface disinfected by placing in 70% ethanol for 3 minutes to break surface tension, followed by 10 minutes in 1% sodium hypochlorite for disinfection, and three rinses in sterile water, and then patted dry on sterile tissue paper. Six root pieces taken at random from each plant were plated on to media, with two root pieces per Petri plate for a total of three plates for each of the 32 plant samples. Media consisted of acidified potato dextrose agar (APDA) containing 1 ml of 10% lactic acid per 100 ml media to hinder bacterial growth, following manufacturer's instructions (Oxoid Ltd, Thermo Fisher Scientific, Basingstoke, England). To confirm that surface disinfection was successful, one disinfected root piece from each site was drawn over the surface of media. No fungal growth occurred on these plates.

Plates were sealed individually with Parafilm, incubated at 23˚C, and checked daily for three weeks for fungal growth. Fungi that grew from the roots were isolated into pure culture on fresh plates of APDA. All disinfection, plating, and isolations were conducted in a biological safety cabinet using aseptic techniques (Forma Class II, A2, Thermo Electron Corporation). Plates were rearranged each day to mitigate potential differences in temperature at different locations in the incubator. Pure cultures were categorised into morphotypes based on colony characteristics such as colour and texture to aid identification.

4.3.1.2. Identification of fungi

4.3.1.2.1. Identification via DNA extraction, amplification, and sequencing

All isolates were identified to genus or species by using a combination of DNA sequencing and morphology to mitigate biases associated with each technique (Porras-Alfaro and Bayman, 2011). One fungal isolate from each morphotype was grown for DNA extraction, except one morphotype for which three isolates were sequenced due to morphological variability in the cultures. Morphotypes were cultured on acidified potato dextrose broth (Atlas, 2010), acidified using the same method as the APDA. A plug from each pure culture was inoculated into 30 ml of broth in individual 50 ml sterile falcon tubes (BD, Franklin Lakes, NJ, USA). Falcon tubes were secured in an incubator shaker at 23˚C and 200 RPM. After one week, approximately 300 mg of mycelia from each falcon tube was placed into sterile 2 ml microcentrifuge tubes containing sterile 3 mm tungsten carbide beads. Fungal tissue was homogenised by shaking the tubes at 30 mHz for 10 minutes in a MoBio 96 Well Plate Shaker (MoBio Laboratories, Carlsbad, CA, USA). Subsequent DNA extraction was conducted using the Macherey-Nagel Nuceleospin Mini kit using cell CTAB lysis buffer (Macherey-Nagel and Co. KG, Düren, Germany).

Extracted DNA was amplified by PCR using fungal barcoding primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC; White et al., 1990), as recommended by Schoch et al. (2012). The PCR mix was composed of 500 nM of each primer, 4 µl 5X buffer 25 mM magnesium chloride 200 nM dinucletotide triphosphate mix 1.25 units of GoTaq Flexi polymerase enzyme (Promega, Madison, WI, USA), and 1 µl template fungal DNA. Total volume of the mix was made up to 20 µl with sterile DNase free water. The

PCR program consisted of an initial cycle of DNA denaturation at 95°C for 10 minutes, followed by 35 cycles of 95°C for 15 seconds, annealing at 49.5°C for 30 seconds, elongation at 72°C for 90 seconds, followed by a 7 minute elongation at 72°C. PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Saint Louis, MO, USA) and sequenced by Sanger sequencing (Sanger et al., 1977) at the Genomics Facility in the Advanced Analysis Centre at the University of Guelph (http://www.uoguelph.ca/~genomics/). Identities of sequences were entered into the BLAST tool to find closely related sequences in GenBank (Altschul et al., 1990; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.1.2.2. Identification via morphological characteristics

Identifications of all sequenced and unsequenced fungal isolates of each morphotype were verified using morphological traits, aided by the identities that closely matched in Genbank. Cultures that would not sporulate on APDA were placed under UV, plated onto V8 media (Atlas, 2010), or “wounded” with a sterile toothpick which was then placed on the media to induce sporulation. Identification was greatly aided by knowledge and advice from Professor Emeritus G. J. Boland (University of Guelph, ON, Canada). Voucher specimens of each isolate are available in long-term culture collections both at Algoma University (P. M. Antunes) and the University of Guelph (K. E. Dunfield).

Once all isolates were identified, I searched for any publications reporting them as a pathogen by individually searching each fungal name AND “pathog*” in the Web of Science in August 2014. I also searched for previous records of each fungus on *V. rossicum* using both the Web of Science and the United States Department of Agriculture fungus-host distribution database (http://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm). All searches were repeated with *Cynanchum*, a common synonym of *Vincetoxicum*.

4.3.2. Pathogenicity trial

Two pathogenicity trials were run: Trial 1 was used to assess growth effects and the ability of seven fungi to colonise *V. rossicum*. In Trial 2, three fungi that had high rates of recovery from *V. rossicum* roots in Trial 1 were tested on *V. rossicum*, *A. syriaca*, and *S. canadensis*.

4.3.2.1. Trial 1: testing colonisation and growth effects of seven fungi on V. rossicum

Seven fungal isolates that are known pathogens on other plant species were chosen to test their growth effects on *V. rossicum*: *Cadophora* sp. (GR BOTAO1), *Colletotrichum* sp. (GC SAURO1), *Ilyonectria* sp. (YE BOTAO4), *Ilyonectria radicicola* (RE OMEMY1), *Macrophomina phaseolina* (BL HAMIY4), *Phaeocytostroma* sp. (MN ROUGO3), and *Phomopsis* sp. (MR ROUGO3; Tables 4.1 and S 4.1). *Vincetoxicum rossicum* seeds (collected from Charles Sauriol Reserve in the Don Valley, Toronto, ON, Canada) were stratified between sheets of moist filter paper and stored at 4˚C for one week and then surface disinfected using the same method as the root disinfection. Seeds were pregerminated in 1:1:1 sterile soil:turface:sand mix in individual trays for each species for six weeks at 14/10 hours day/night with temperatures 23˚C and 18˚C, 500 µmol light, and 60% relative humidity.

Fungal-inoculated rye seeds were used as inoculum and mixed with substrate, using methods advised by Professor Emeritus G. J. Boland. Pure cultures of each isolate were grown on plates of APDA at 23˚C. A volume of 500 ml of untreated rye seeds were mixed with 500 ml deionised water and left to soak for one hour in an autoclave bag. Rye seeds were then autoclaved for one hour and left to cool at room temperature for at least five hours. To inoculate, a 5 cm diameter of the pure culture was cut into approximately 1 cm squares with a flame-sterilised scalpel and mixed into each the bags of rye seed under aseptic conditions in a biological safety cabinet. Control bags were not inoculated. Fungi grew on the grain in these bags for two weeks at room temperature and bags were mixed thoroughly every two days to ensure maximum colonisation of the seeds. Seeds were then dried on bleach-disinfected mesh screens in a growth chamber for three days. Dried seeds were ground using a food grinder, which was disassembled and thoroughly washed and soaked in hot water with bleach, rinsed, and dried between grinding each isolate. These inocula were stored in paper bags at 10˚C. To

confirm fungal viability and that inocula were pure cultures, each inoculum was plated on ADPA and incubated at 23˚C for one week prior to potting the experiment; fungal-inoculated seeds were in pure cultures and there was no growth from controls after seven days.

There were nine replicates for each of the nine plant-fungus treatment combinations: *Cadophora* sp., *Colletotrichum* sp., *Ilyonectria* sp., *I. radicicola*, *M. phaseolina*, *Phaeocytostroma* sp., *Phomopsis* sp., uninoculated control, and mix of all seven fungi (hereafter referred to as Mix) for a total of 81 experimental units. Sieved soil, sand, and turface were mixed in a 1:1:1 ratio, autoclaved twice over three days (90 minutes at 121ºC and 18 psi), and then stored at room temperature for one week to allow mineralisation. The inoculum for each fungal treatment was mixed into this substrate at a rate of five grams per litre and placed into 1 L pots lined with mesh and placed on saucers to minimise nutrient losses. The Mix treatment contained 0.7 g of each isolate per litre (0.7 g × 7 isolates = 5 g per L total). A 1 cm layer of sterile sand was placed on the top of each pot to prevent cross-contamination through airborne particles. All equipment for potting and containers had been previously soaked in bleach, rinsed and dried. For each plant-fungal combination, one seedling was transplanted into each pot and replaced after one week if they did not survive. Growth chamber settings were 14/10 hours day/night with temperatures 23˚C and 18˚C, 500 µmol light, and 60% relative humidity. Plants were watered one to three times a week and re-randomised monthly. Plants were given low levels of fertiliser nine and ten weeks after planting (20:20:20 N:P:K, 30 ppm, Plant Products, Brampton, ON, Canada).

Plants were harvested after 11 weeks of growth because this is considered a reasonable period to observe pathogenic effects. A total of 66 experimental units survived the experiment. At least one replicate of *V. rossicum* in each fungal treatment, except for the Mix, did not survive the experiment: one replicate died from each of *Cadophora* sp., *Ilyonectria* sp., *M. phaseolina*, and *Phaeocytostroma* sp.; two were lost from *Colletotrichum* sp.; and three were lost from each of *Phomopsis* sp., *I. radicicola*, and the uninoculated controls. Shoots were separated from roots to obtain separate measures of biomass. Roots were thoroughly washed in tap water and patted dry with paper towels. Two subsamples of roots were taken: one subsample was surface disinfected to reisolate fungi and the other was stored in 70% ethanol to stain and assess fungal colonisation. The roots were then re-weighed to obtain fresh weight to be able to back-calculate root biomass. These were surface disinfected by 30 seconds in 70% ethanol, two minutes in 1%

sodium hypochlorite and three rinses in sterile water. Root pieces were patted dry with sterile tissue and placed on to APDA plates for incubation at 23˚C. Surface disinfection times were shorter for this compared to the field roots because the roots from the growth chamber were considerably smaller and more delicate. Plates where surface disinfected root pieces had been drawn across the media showed no growth. Remaining roots and shoots were dried at 60˚C for at least three days then weighed. All plants were weighed twice to ensure consistency.

4.3.2.2. Trial 2: testing growth effects of three fungi on V. rossicum, A. syriaca*, and* S. canadensis

Three isolates from the first trial that had high rates of recovery from *V. rossicum*, *M. phaseolina* (BL HAMIY4), *I. radicicola* (RE OMEMY1), and *Cadophora* sp. (GR BOTAO1; Table S 4.2), were used in a subsequent pathogenicity test that included two native plants, *A. syriaca* and *S. canadensis*. Viability and purity of stored inocula from Trial 1 was again confirmed by plating on ADPA and incubating at 23˚C for one week prior to potting the experiment. Seeds of *V. rossicum* (collected from Charles Sauriol Reserve in the Don Valley, Toronto, ON, Canada), *A. syriaca* (Richters herbs, ON, Canada), and *S. canadensis* (P. Carson, Norfolk County, ON, Canada) were stratified, surface disinfected, and pre-germinated in the same conditions as Trial 1.

There were six replicates for each of the five plant-fungus treatment combinations: *Cadophora* sp., *I. radicicola*, *M. phaseolina*, uninoculated control, and mix of all three fungi (Mix) for a total of 90 experimental units. Substrate was prepared in the same way and inocula was added at the same rate as in Trial 1; the Mix treatment contained 1.7 g of each isolate per litre (1.7 g × 3 isolates = 5 g per L total). Growth chamber settings and watering regime were the same as in Trial 1, except that low levels of fertiliser were applied once a week (20:20:20 N:P:K, 30 ppm N, Plant Products, Brampton, ON, Canada). Plants were harvested after 11 weeks of growth using the same protocols as in Trial 1. Two individuals of *V. rossicum* and *A. syriaca* did not survive the experiment; one died in the control and one died in the Mix for each plant species, resulting in a total of 87 experimental units. To ensure colonisation of the inoculated fungi, three random replicates from each treatment were selected for root subsampling, surface-disinfection, and plated on to APDA to confirm colonisation.

4.3.3. Data Analysis

All statistical analyses were run in R v. 3.1.0 (R Core Development Team, 2014). For each trial, I ran separate linear models for each of three response variables: aboveground biomass, total biomass, and root-shoot ratio. Fungal isolate was the predictor variable. Pre-planned treatment contrasts were specified where the effect of each fungal treatment is compared to the uninoculated controls. For Trial 1, all root mass was used for reisolation or staining for all replicates of *I. radicicola* and *Colletotrichum* sp., so the models for total biomass and root-shoot ratio do not include these. For Trial 1, aboveground biomass was square-root transformed, and total biomass was log-transformed. For Trial 2, aboveground and total biomass for *V. rossicum* were square-root transformed, and root-shoot ratio was log transformed to meet the assumption of normality.

4.4. Results

4.4.1. Experiment 1: Isolation of fungi from roots

A total of 18 unique fungal taxa were isolated from surface disinfected field roots of *V. rossicum* (Tables 4.1 and S 4.1). Of these 15 have been recorded as pathogenic on other plant species. All but two of the 18 have not been recorded on other *Vincetoxicum* species (Table 4.1). The most frequently isolated fungus was *Monographella cucumerina*, which was isolated from six plants. Across all eight sites, at least one fungal isolate was isolated from 20 of the 32 plants; i.e., no fungi grew from the roots of 12 plants.

4.4.2. Experiment 2: Pathogenicity trials

4.4.2.1. Trial 1: testing colonisation and growth effects of seven fungi on V. rossicum

Vincetoxicum rossicum plants inoculated with *M. phaseolina* had significantly higher aboveground biomass and total biomass compared to the uninoculated controls (Tables 4.2 and 4.3; Fig. 4.1). Plants inoculated with *Phaeocytostroma* sp. also showed significantly higher total biomass (Table 4.3, Fig. 4.1) and marginally significantly higher root-shoot ratio compared to the controls (Table 4.4, Fig. 4.1). Plants inoculated with *Cadophora* sp. and the Mix of all seven fungi had significantly higher root-shoot ratios than the uninoculated control, and the effect of *Ilyonectria* sp. was marginally significant in the same direction (Table 4.4, Fig. 4.1). All inoculated fungi were able to be reisolated from at least one replicate (Table S 4.3).

Table 4.1 Fungal taxa isolated from roots of 32 *V. rossicum* plants from eight sites, in terms of the number of plants from which they were isolated, whether they have been reported as a pathogen, and if they have been reported on *Vincetoxicum* sp. References are provided in brackets.

Fungus	Number of plants	Reported as pathogen?	Previously reported on *Vincetoxicum* sp.?
Alternaria sp	1	Yes, leaf spot, blight (Couture et al., 2003)	No
Cadophora sp.	4	Yes, brown stem rot (Harrington and McNew, 2003; Di Marco et al., 2004) and Petri disease (Gramaje et al., 2011)	Yes, *V. rossicum* (Bongard et al., 2013)
Cadophora orchidicola	1	No	No
Colletotrichum sp.	5	Yes, anthracnose (Fitzell and Peak, 1984; Freeman and Katan, 1997; Afanador-Kafuri et al., 2003; Farr et al., 2006; Anderson et al., 2013)	Yes, *Colletotrichum lineola* isolated from *V. scandens* and caused anthracnose on *V. rossicum, V. nigrum* and *V. scandens* (Berner et al., 2011)
Colletotrichum destructivum	1	Yes, anthracnose (Sun and Zhang, 2009; Tomioka et al., 2012)	No
Eupenicillium euglaucum	1	No	No
Geniculosporium sp.	2	Yes, necrosis (Causin et al., 2004)	No
Harpophora graminicola	2	Yes, blight (Smiley and Fowler, 1984)	No
Ilyonectria sp.	1	Yes, black foot disease (Cabral et al., 2012a)	No
Ilyonectria radicicola	5	Yes, root rot (Cabral et al., 2012b)	No

Table S 4.1 continued.

Fungus	Number of plants	Reported as pathogen?	Previously reported on *Vincetoxicum* sp.?
Macrophomina phaseolina	1	Yes, charcoal rot and ashy stem blight diseases (Mihail and Taylor, 1995; Aegerter et al., 2000; García-Jiminéz et al., 2000)	No
Monographella cucumerina	6	Yes, root and collar rots (Andrade-Linares et al., 2011; Carlucci et al., 2012)	No
Phaeocytostroma sp.	1	Yes, root rot (Lamprecht et al., 2011)	No
Paraphoma sp.	1	Yes (de Gruyter et al., 2010)	No
Paraphoma chrysanthemicola	1	Yes, bud browning and wilting (Zolna et al., 2013)	No
Penicillium camemberti	1	No	No
Phomopsis sp.	1	Yes, blight, dieback (Couture et al., 2003)	No
Rhizopycnis vagum	1	Yes, root rot (Aegerter et al., 2000; Biernacki and Bruton, 2001; Armengol et al., 2003)	No

Table 4.2 Coefficient estimates, *t*-statistics, and *P* values for each fungal treatment from linear model of square-root transformed aboveground biomass of *V. rossicum* in Trial 1. Standard errors are in brackets.

Treatment	Coefficient (standard error)	*t*	*P*
Uninoculated control (intercept)	0.173 (0.021)	8.362	<0.001
Cadophora sp.	-0.029 (0.027)	-1.048	0.299
M. phaseolina	0.076 (0.027)	2.773	0.008
Ilyonectria sp.	-0.025 (0.027)	-0.912	0.365
Phaeocytostroma sp.	0.050 (0.027)	1.812	0.075
Phomopsis sp.	-0.000 (0.029)	-0.017	0.987
Colletotrichum sp.	-0.000 (0.028)	-0.303	0.763
I. radicicola	-0.008 (0.029)	-0.286	0.776
Mix	-0.012 (0.027)	-0.440	0.662

Note: Coefficient estimates represent pre-planned comparisons of differences between the mean of each treatment mean and the mean of the uninoculated control.

Table 4.3 Coefficient estimates, *t*-statistics, and *P* values for each fungal treatment from linear model of log transformed total biomass of *V. rossicum* in Trial 1. Standard errors are in brackets.

Treatment	Coefficient (standard error)	*t*	*P*
Uninoculated control (intercept)	-2.509 (0.176)	-14.254	<0.001
Cadophora sp.	0.178 (0.269)	0.663	0.513
M. phaseolina	0.927 (0.227)	4.080	<0.001
Phomopsis sp.	-0.028 (0.269)	-0.105	0.917
Phaeocytostroma sp.	0.656 (0.221)	2.971	0.006
Ilyonectria sp.	-0.112 (0.236)	-0.474	0.639
Mix	0.038 (0.227)	0.165	0.870

Note: Coefficient estimates represent pre-planned comparisons of differences between the mean of each treatment mean and the mean of the control.

Table 4.4 Coefficient estimates, *t*-statistics, and *P* values for each fungal treatment from linear model of root-shoot ratio of *V. rossicum* in Trial 1. Standard errors are in brackets.

Treatment	Coefficient (standard error)	*t*	*P*
Uninoculated control (intercept)	0.163 (0.201)	0.814	0.423
Cadophora sp.	0.645 (0.307)	2.105	0.045
M. phaseolina	0.356 (0.259)	1.375	0.180
Ilyonectria sp.	0.539 (0.269)	2.000	0.056
Phaeocytostroma sp.	0.505 (0.252)	2.006	0.055
Phomopsis sp.	0.164 (0.307)	0.536	0.596
Mix	0.728 (0.259)	2.808	0.009

Note: Coefficient estimates represent pre-planned comparisons of differences between the mean of each treatment mean and the mean of the control.

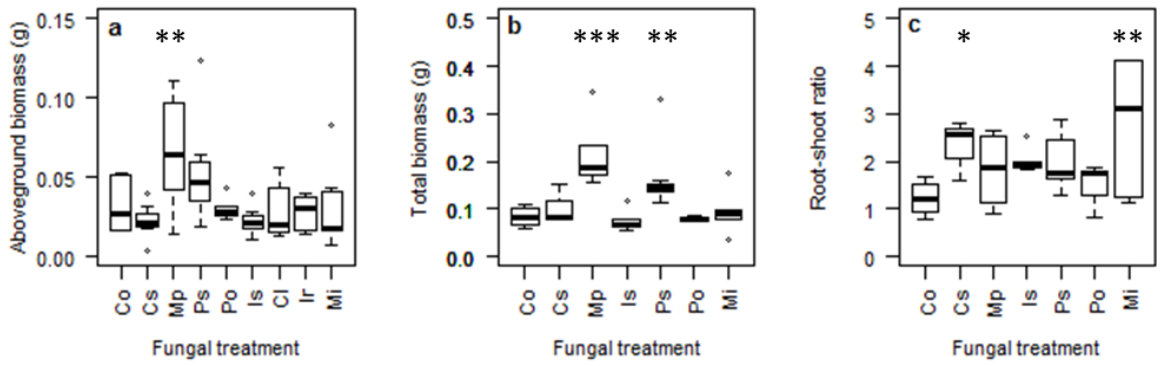

Fig. 4.1 Effect of fungal treatments on *V. rossicum*'s (a) aboveground biomass (g), (b) total biomass (g), and (c) root-shoot ratio in Trial 1. Co = uninoculated control, Cs = *Cadophora* sp., Mp = *M. phaseolina,* Is=Ilyonectria sp., Ps = *Phaeocytostroma* sp., Po = *Phomopsis* sp., Cl = *Colletotrichum* sp., Ir = *I. radicicola*, Mi = Mix of all seven fungi. The box represents the interquartile range, the band represents the median, and the whiskers indicate data within 1.5 times the interquartile range. Points are outliers beyond the whisker range. Statistically significant differences compared to the controls are indicated by asterisks (P* <0.05, ** *P* <0.01, ** *P* <0.01. Tables 4.2-4.4).**

4.4.2.2. Trial 2: testing growth effects of three fungi on V. rossicum, A. syriaca, and S. canadensis

Each of the three plant species responded differently to the fungal treatments. Pre-planned contrasts in the linear models showed that, relative to the controls, there were no differences in aboveground biomass for any plant species (Table 4.5, Fig. 4.2). Total biomass of *V. rossicum* was higher when inoculated with *I. radicicola*, *M. phaseolina*, and the Mix (Table 4.6, Fig. 4.2). In addition, all fungal treatments had higher root-shoot ratios in *V. rossicum* plants compared to controls (Table 4.7, Fig. 4.2). In contrast, the Mix reduced the total biomass of *S. canadensis*, whereas each individual fungal isolate had no effect (Table 4.6, Fig. 4.2). *Ascelpias syriaca* showed no significant differences in total biomass in any fungal treatment compared to controls (Table 4.6, Fig. 4.2). There were no significant differences in root-shoot ratios between fungal treatments and control plants for either *A. syriaca* or *S. canadensis* (Table 4.7, Fig. 4.2).

Table 4.5 Coefficient estimates, *t*-statistics, and *P* values for each fungal treatment from linear models for aboveground biomass of *V. rossicum, A. syriaca,* and *S. canadensis* in Trial 2. Standard errors are in brackets. Data for *V. rossicum* were square-root transformed.

Plant species	Fungal treatment	Coefficient (standard error)	*t*	*P*
V. rossicum	Uninoculated control (Intercept)	0.18 (0.03)	5.78	<0.001
	Cadophora sp.	-0.03 (0.04)	-0.67	0.511
	I. radicicola	0.02 (0.04)	0.422	0.677
	M. phaseolina	0.06 (0.04)	1.453	0.160
	Mix	0.01 (0.04)	0.16	0.87
A. syriaca	Uninoculated control (Intercept)	-0.59 (0.05)	10.84	<0.001
	Cadophora sp.	-0.03 (0.07)	-0.41	0.686
	I. radicicola	0.00 (0.07)	0.02	0.981
	M. phaseolina	0.03 (0.08)	0.36	0.726
	Mix	0.00 (0.08)	-0.06	0.951
S. canadensis	Uninoculated control (Intercept)	1.34 (0.13)	10.75	<0.001
	Cadophora sp.	0.02 (0.18)	0.12	0.906
	I. radicicola	0.05 (0.18)	0.30	0.763
	M. phaseolina	0.22 (0.18)	1.22	0.234
	Mix	-0.33 (0.18)	-1.85	0.076

Note: Coefficient estimates represent pre-planned comparisons of differences between the mean of each treatment mean and the mean of the control.

Table 4.6 Coefficient estimates, *t*-statistics, and *P* values for each fungal treatment from linear models for total biomass for *V. rossicum, A. syriaca,* and *S. canadensis* in Trial 2. Standard errors are in brackets. Data for *V. rossicum* were square-root transformed.

Plant species	Fungal treatment	Coefficient (standard error)	*t*	*P*
V. rossicum	Uninoculated control (Intercept)	0.24 (0.05)	5.33	<0.001
	Cadophora sp.	0.11 (0.06)	1.81	0.083
	I. radicicola	0.18 (0.06)	2.93	0.008
	M. phaseolina	0.26 (0.06)	4.27	<0.001
	Mix	0.15 (0.06)	2.38	0.026
A. syriaca	Uninoculated control (Intercept)	1.41 (0.26)	5.44	<0.001
	Cadophora sp.	-0.31 (0.35)	-0.88	0.388
	I. radicicola	0.11 (0.35)	0.33	0.746
	M. phaseolina	0.22 (0.35)	0.63	0.538
	Mix	0.01 (0.37)	0.03	0.976
S. canadensis	Uninoculated control (Intercept)	2.86 (0.23)	12.70	<0.001
	Cadophora sp.	-0.12 (0.32)	-0.37	0.713
	I. radicicola	0.12 (0.32)	0.38	0.710
	M. phaseolina	0.24 (0.32)	0.74	0.466
	Mix	-0.90 (0.32)	-2.83	0.009

Note: Coefficient estimates represent pre-planned comparisons of differences between the mean of each treatment mean and the mean of the control.

Table 4.7 Coefficient estimates, *t*-statistics, and *P* values for each fungal treatment from linear models for root-shoot ratio of *V. rossicum, A. syriaca,* and *S. canadensis* in Trial 2. Standard errors are in brackets. Data for *V. rossicum* were log transformed.

Plant species	Fungal treatment	Coefficient (standard error)	*t*	*P*
V. rossicum	Uninoculated control (Intercept)	-0.20 (0.40)	-0.51	0.617
	Cadophora sp.	1.56 (0.54)	2.87	0.009
	I. radicicola	1.48 (0.54)	2.72	0.012
	M. phaseolina	1.43 (0.54)	2.64	0.015
	Mix	1.30 (0.57)	2.30	0.031
A. syriaca	Uninoculated control (Intercept)	2.98 (0.45)	6.58	<0.001
	Cadophora sp.	-0.44 (0.61)	-0.72	0.477
	I. radicicola	0.51 (0.61)	0.84	0.411
	M. phaseolina	0.86 (0.61)	1.40	0.174
	Mix	0.33 (0.64)	0.51	0.615
S. canadensis	Uninoculated control (Intercept)	1.14 (0.08)	13.56	<0.001
	Cadophora sp.	-0.11 (0.12)	-0.93	0.364
	I. radicicola	0.01 (0.12)	0.11	0.914
	M. phaseolina	-0.13 (0.12)	-1.09	0.285
	Mix	-0.20 (0.12)	1.72	0.099

Note: Coefficient estimates represent pre-planned comparisons of differences between the mean of each treatment mean and the mean of the control.

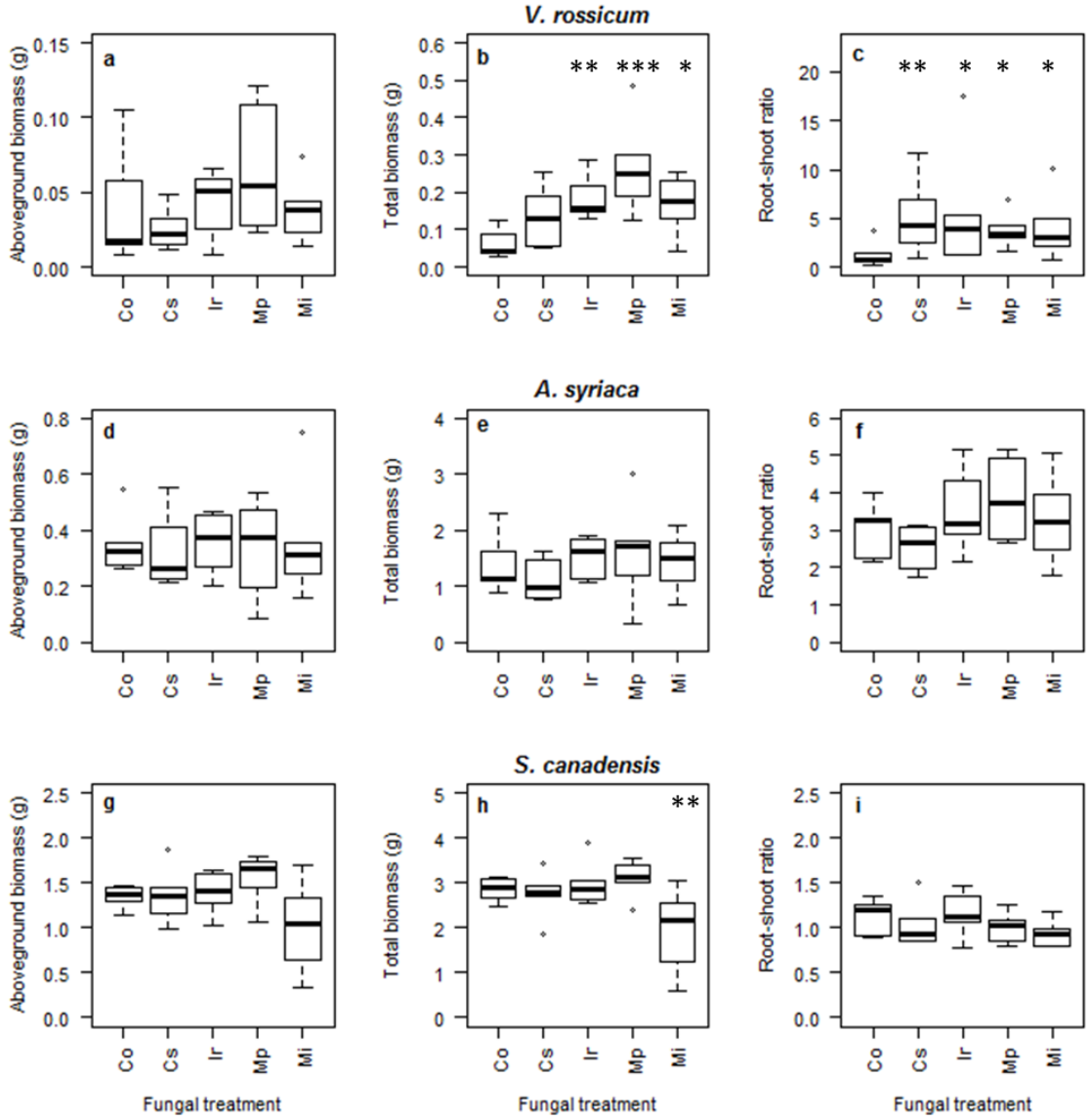

Fig. 4.2 Caption on next page.

Fig. 4.2 Caption: Effect of fungal treatments on *V. rossicum'*s (a) aboveground biomass (g), (b) total biomass (g), and (c) root-shoot ratio, *A. syriaca*'s (d) aboveground biomass (g), (e) total biomass (g), and (f) root-shoot ratio, and *S. canadensis*' (g) aboveground biomass (g), (h) total biomass (g), and (i) root-shoot ratio in Trial 2. Co = uninoculated control, Cs = *Cadophora* sp., Mp = *M. phaseolina,* Is=*Ilyonectria* sp., Ps = *Phaeocytostroma* sp., Po = *Phomopsis* sp., Cl = *Colletotrichum* sp., Ir = *I. radicicola*, Mi = Mix of all seven fungi. The box represents the interquartile range, the band represents the median, and the whiskers indicate data within 1.5 times the interquartile range. Points are outliers beyond the whisker range. Statistically significant differences compared to the controls are indicated by asterisks (P* <0.05, ** *P* <0.01, ** *P* <0.01. Tables 4.5-4.7).**

4.5. Discussion

Results supported the hypothesis that *V. rossicum* associates with many culturable root-associated fungi. Many represented new records in this genus (Table 4.1). I isolated 18 unique taxa of which 15 are known pathogens on other plant species (Table 4.1). In agreement with my hypothesis, none of the tested pathogens reduced the growth of *V. rossicum* in either of the two trials. In fact, five of the seven fungi that are known pathogens on other plant species increased growth of *V. rossicum* in some way. The other two, *Phomopsis* sp. and *Colletotrichum* sp., did not significantly reduce growth. These results demonstrate that *V. rossicum* associates with fungi that increase its growth. The hypothesis that the fungi would be more pathogenic on *A. syriaca* because it is in the same family as *V. rossicum* was not supported. This plant showed neither increased nor reduced growth in the presence of any of the three isolates tested on this plant species (Tables 4.5-4.7, Fig. 4.2). The only pathogenic response observed over both trials was a reduction in total biomass when *S. canadensis* was inoculated by the Mix of three fungi, but they had no effect individually (Table 4.6), suggesting synergistic interactions or cumulative effects of multiple fungi.

Fungi isolated from *V. rossicum* roots in the field that were shown to increase its own biomass and root-shoot ratio, and were also able to colonise and reduce growth of a native species with which *V. rossicum* commonly co-occurs, *S. canadensis* (Tables 4.2, 4.3, 4.5, S 4.2, and S 4.3). These data suggest two theories regarding species invasions: firstly, invasive species may act as reservoirs for biota that are pathogenic on native species (Daszak et al., 2000; Power and Mitchell, 2004; Li et al., 2014), and secondly, invasive species may mediate plant community interactions and competition via soil biota by associating with organisms that are more pathogenic to co-occurring native species than themselves (Colautti et al., 2004; Eppinga et al., 2006; Mangla et al., 2008; Beckstead et al., 2010). Previous work has shown that *V. rossicum* outcompetes *S. canadensis* and that this may be mediated by soil biota (Sanderson and Antunes, 2013). Pathogen transfer is more likely to occur if an invasive plant is an effective pathogen reservoir and is in high densities (Power and Mitchell, 2004; Malmstrom et al., 2005; Gilbert and Parker, 2006). This may be more important for determining plant community structure than having a diversity of plants harbouring multiple pathogens. For example, *Avena fatua* is an effective reservoir of barely yellow dwarf virus, and surrounding species experience higher pathogen loads when it is present compared to when it is absent in plant communities (Power and Mitchell, 2004).

There were host-specific responses of each fungal treatment for each plant species (Tables 4.5-4.7, Fig. 4.2). These fungi that are known pathogens on other plant species increased *V. rossicum*, reduced or had no effect on *S. canadensis*, and had neutral effects on *A. syriaca*. The grain inoculum was well colonised and in pure culture when mixed with the substrate. However, many fungi were isolated from seeds of *V. rossicum* and *A. syriaca* (Table S 4.4), which may explain the large numbers of non-inoculated fungi that were reisolated from some roots (Tables S 4.2 and S 4.3). Vertically-transmitted fungi may be less likely to be pathogenic because they are more reliant on the host for fitness (Rodriguez et al., 2009). Pathogens may increase growth in some plants because they induce modes of plant defence that are shared with plant-growth promoting fungi (Bent, 2006; Doehlemann et al., 2014). For example, a change in just one gene or enzyme can be enough to change a saprophyte to a pathogen and allow host switching (Schäfer et al., 1989; James et al., 2006). In relation to the fungi

tested here, *Cadophora malorum* is often pathogenic across many host species (Harrington and McNew, 2003; Di Marco et al., 2004; Gramaje et al., 2011), but one isolate in South Korea has been shown to produce giberellins that promotes growth of two plant species (You et al., 2013). *Macrophomina phaseolina* is a well-studied pathogen with a wide range of hosts. A comprehensive pathogenicity test across two continents found that at least one of 114 strains caused pathogenic effects on at least one of the four crop species tested (Mihail and Taylor, 1995). The observation that this fungus acted as a plant-growth promoter on *V. rossicum* across both trials was therefore surprising (Tables 4.2-4.7, Figs. 4.1 and 4.2). It is possible that non-domesticated plants are more resistant to crop pathogens because they have higher genetic diversity. For example, Mitchell et al. (2010) found that introduced exotic plants with a history of agricultural use had higher pathogen richness.

I had expected that growth of *V. rossicum* would not be affected by the fungal isolates that are pathogenic in other plant species, but instead they increased its growth (Tables 4.2-4.7, Figs. 4.1 and 4.2). *Vincetoxicum rossicum* associates with many AM fungal species and gains a net benefit from associating with soil biota, even when there are many fungi that are known pathogens on other plant species present (Smith et al., 2008; Bongard et al., 2013; this thesis chapters 2 and 3). Cumulatively, these studies suggest that soil biota are important for the growth of this species and that it may have few enemies in its invasive range. In addition, novel weapons, perhaps via (-)-antofine, could reduce *V. rossicum*'s susceptibility to North American pathogens (Callaway et al., 2005; this thesis chapter 3). The combination of recruiting plant growth promoters, positive feedback, and acting as a reservoir for pathogens of a common co-occurring native plant, are plausible mechanisms that may explain how *V. rossicum* maintains dominance in plant communities its invasive range. Further work testing the competitive interactions of these plant species in the presence of known fungi would help to elucidate the importance of these organisms in mediating plant interactions.

This study is useful for isolating the effects of individual taxa on three co-occurring plant species. However, realised growth responses of plants to fungi are undoubtedly influenced by interactions between pathogens and other biota (Morris et al.,

2007; van der Putten et al., 2007a; Sikes et al., 2009; Rillig et al., 2014). The potential effects of these interactions and potential synergisms are emphasised by the fact that biomass of *S. canadensis* was only reduced when inoculated with all three fungi, but not individually (Table 4.6, Fig. 4.2). This could be important because the native plants tested here have previously been shown to interact strongly with soil microbes. *Solidago canadensis* benefits from soil biota and generates positive feedback with AM fungi in areas where it is dominant in China and the USA (Sun and He, 2010; Zhang et al., 2010; Pendergast et al., 2013). Similarly, *A. syriaca* can suppress root-feeding herbivores via interactions between root exudates and soil biota (Rasmann et al., 2011). In addition, colonisation by one fungal pathogen can increase susceptibility to the effects of others, or less virulent strains may be replaced by more competitive strains (Jarosz and Davelos, 1995). The relative importance of pathogen interactions for determining plant community structure compared to other mechanisms is an area that requires more research (Mordecai, 2011). In addition, knowing the relative abundance of each fungus that colonised the root, for example via quantitative PCR, would allow greater understanding of how abundances of different fungi that colonise change in the presence of other fungi.

Enemy release may be an important mechanism for the success of introduced plants at large, biogeographical scales, but perhaps not always at local scales (Colautti et al., 2004). It was expected that plant phylogeny and origin would be good predictors of plant growth in that the fungi from exotic *V. rossicum* plants would reduce growth of the native *A. syriaca*. This hypothesis was not supported (Tables 4.5-4.7, Fig. 4.2), but it is difficult to generalise from one phylogenetically matched pair of exotic and native origin. However, a comprehensive study testing phylogeny and origin of plants and fungal pathogens also did not show higher infection and virulence on native compared to exotic hosts (Gilbert and Parker, 2010). Other studies have also shown inconsistent responses of native species to pathogens on exotic species (Beckstead et al., 2010; Flory et al., 2011). It is possible that the strains of fungi tested here were not pathogenic on plant species within Apocynaceae. If resistance to pathogens is phylogenetically conserved, then exotic species in the same family as the natives could be ‘pre-adapted’ to the enemies in the novel range, and could explain why exotic species that are more closely

related to the natives are more likely to establish in some communities (e.g., Duncan and Williams, 2002; Shea and Chesson, 2002). This is the opposite of what is expected under the hypothesis of limiting similarity (MacArthur and Levins, 1967). In this respect, *A. syriaca* could be facilitating *V. rossicum* in some way. In fact, *A. syriaca* itself is considered a noxious weed in some parts of Ontario and Quebec (Bhowmik and Bandeen, 1976; http://www.omafra.gov.on.ca/english/crops/facts/info_milkweed.htm). Further research incorporating multiple plants from different families would help elucidate the importance of phylogeny and pathogenic effects.

Few studies have isolated and tested the growth effects of root-associated fungi on invasive plants (e.g., Holah and Alexander, 1999; Mangla et al., 2008; Rillig et al., 2014). The ability to isolate many fungi from surface-disinfected roots and seeds of *V. rossicum* was expected based on previous sequencing data (this thesis chapter 3), but these records are further strengthened by combining sequences with morphological identification. Many of these fungi have not been previously recorded on *V. rossicum* or its relatives and are known pathogens of other plant species (Table 4.1). Since most of the isolated fungi were identified as fungi that are known pathogens on other plant species, it is possible that this criterion was too broad to assess pathogen accumulation. The well-studied disease triangle emphasises that pathogenic effects may only be observed in specific combinations of host and fungal genotypes and environmental conditions (Agrios, 2005; Gilbert and Parker, 2006; Beckstead et al., 2010). It is also possible that organs or tissues other than the roots of *V. rossicum* are susceptible to one or more of these pathogens (Dhingra et al., 2002; Aly et al., 2011). For example, many of the pathogens isolated cause disease on aboveground tissues (Table 4.1), so their function in the soil could be quite different. I used fungi isolated from the invasive range and climate settings typical of a Toronto summer, where *V. rossicum* is abundant (Miller et al., 2007; Sanderson and Antunes, 2013), so I consider this to have been realistic if pathogen symptoms were to occur. Furthermore, these results were consistent across two trials. It is also possible that less virulent strains were inadvertently selected for by using a generalist media (Agrios, 2005; Morris et al., 2007; Aly et al., 2011). However, other studies have demonstrated pathogenic effects of root-associated fungi isolated on PDA (e.g., Holah and Alexander, 1999). Investigating functional responses to these fungi

over a range of environmental conditions may further clarify where they fall along the growth-promoting and growth-reducing continuum and, consequently, under which conditions they may mediate *V. rossicum* dominance.

4.6. Conclusions

These results show that *V. rossicum* associates with fungi that increase its growth. In addition, some of these fungi can reduce growth of the co-occurring native *S. canadensis*, which could lead to microbially-mediated apparent competition between these two plant species. It is possible that *V. rossicum* may act as a pathogen reservoir in the field to maintain dominance in plant communities. While this study indicates that this is a possibility, to understand the importance of pathogen-mediated plant competition in these communities future research needs to investigate these interactions with these fungi in multi-plant and fungal assemblage settings. In addition, we need an understanding of whether these plants and pathogens are at appropriate densities for pathogen spillover to native species to occur. More empirical research is needed on effects of pathogens on plant coexistence and community structure, and the conditions under which these mechanisms are important (Mordecai, 2011).

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4.7. Supplementary material

Table S 4.1 Sequenced fungal isolates from *V. rossicum* roots and their closest related Genbank sequences. Isolate code is the unique identifier used in this study, based on morphotype. Isolates that were sequenced have the length (in number of base pairs) next to their isolate code. "Current nomenclature" for each isolate was obtained from the Index Fungorum database (http://www.indexfungorum.org/). The "identified as" column is the taxonomic identification assigned based on both sequence and morphological identification.

Isolate code (length)	Closest relative in Genbank (accession number)	Query cover	Similarity (Max identity)	Current nomenclature	Identified as
GR HAMIY2 (594)	*Cadophora* sp. (JN859258)	98%	100%	*Cadophora*	*Cadophora* sp.
MU OMEMY1 (593)	*Cadophora* sp. (JN859254)	100%	99%	*Cadophora*	*Cadophora* sp.
GR HAMIY3 (591)	*Cadophora* sp. (JN859252)	100%	99%	*Cadophora*	*Cadophora* sp.
GR BOTAO1 (610)	*Cadophora* sp. (JN859252)	99%	99%	*Cadophora*	*Cadophora* sp.
CO BOTAO2 (557) CO BOTAO4	*Phialophora graminicola* (U17218)	100%	99%	*Harpophora graminicola*	*Harpophora graminicola*
GC SAURO1 (547) GC HAMIY1 GC HAMIY2	*Colletotrichum* cf. *gloeosporioides* (AY539806)	100%	99%	*Colletotrichum gloeosporioides*	*Colletotrichum* sp.†
	Colletotrichum trifolii (AJ301942)	100%	99%	*Colletotrichum trifolii*	
M2 ROUGO3 (539)	*Colletotrichum gloeosporioides* (HM241948)	100%	99%	*Colletotrichum gloeosporioides*	*Colletotrichum* sp.†
PI BOTAO1 (545)	*Colletotrichum destructivum* (EU070911)	100%	99%	*Colletotrichum destructivum*	*Colletotrichum destructivum*

Table S 4.1 continued

Isolate code (length)	Closest relative in Genbank (accession number)	Query cover	Similarity (Max identity)	Current nomenclature	Identified as
PU BRANY3 (547)	*Colletotrichum* sp. (DQ286215)	100%	99%	*Colletotrichum*	*Colletotrichum* sp.
MU KIRKO3 (604)	*Leptodontidium orchidicola* (AF486133)	100%	99%	*Cadophora orchidicola*	*Cadophora orchidicola*
BL HAMIY4 (561)	*Macrophomina phaseolina* (EF570500)	98%	98%	*Macrophomina phaseolina*	*Macrophomina phaseolina*
RE OMEMY1 (506) RE BOTAO4	*Ilyonectria radicicola* (JN129416)	100%	99%	*Ilyonectria radicicola*	*Ilyonectria radicicola*
RE KIRKO1 RE OMEMY2 RE SAURO1	*Neonectria radicicola* (GQ131875)	100%	99%	*Ilyonectria radicicola*	
YE BOTAO4 (525)	*Ilyonectria alcacerensis* (NR121498)	98%	99%	none	*Ilyonectria* sp.
	Ilyonectria macrodidyma (JN859422)	98%	99%	*Ilyonectria macrodidyma*	
	Neonectria sp. (JF429683)	98%	99%	*Neonectria* sp.	
M1 GARNY1 (563) M1 GARNY4	*Nemania* sp. (FJ210522)	98%	99%	*Nemania* Anamorph: *Geniculosporium*	*Geniculosporium sp.*
SU HAMIY1 (523) SU GARNY1 SU ROUGO1 SU ROUGO4 SU SAURO1 SU SAURO2	*Plectosphaerella cucumerina* (KC427067)	98%	100%	*Monographella cucumerina*	*Monographella cucumerina*
MA SAURO4 (517)	*Alternaria* sp. (DQ779787)	96%*	99%	*Alternaria*	*Alternaria* sp

Table S 4.1 continued

Isolate code (length)	Closest relative in Genbank (accession number)	Query cover	Similarity (Max identity)	Current nomenclature	Identified as
MP HAMIY4 (509)	*Paraphoma* sp. (FJ903342)	99%	100%	*Paraphoma*	*Paraphoma* sp.
GR ROUGO4 (514)	*Paraphoma chrysanthemicola* (KF251166)	98%	99%	*Paraphoma chrysanthemicola*	*Paraphoma chrysanthemicola*
MR ROUGO3 (542)	*Phomopsis* sp. (HE774491)	100%	100%	*Phomopsis*	*Phomopsis* sp.
MN ROUGO3 (556)	*Phaeocytostroma plurivorum* (FR748046)	100%	99%	*Phaeocytostroma plurivorum*	*Phaeocytostroma* sp.⌉
M3 HAMIY2 (554)	*Penicillium camemberti* (KF285997)	99%	99%	*Penicillium camemberti*	*Penicillium camemberti*
WH BRANY3 (466)	*Penicillium euglaucum* (JN617699)	100%	99%	*Eupenicillium euglaucum*	*Eupenicillium euglaucum*
M3 HAMIY4 (503)	*Rhizopycnis vagum* (JN859316)	100%	99%	*Rhizopycnis vagum*	*Rhizopycnis vagum*

*there were other records of *Alternaria* that had higher cover but they were unpublished

‡ Multiple records from this reference

†This species group has recently gone through revision, so it is more parsimonious to keep it as sp.

⌉ morphological identification could not determine to species

Table S 4.2 Trial 1 number of *V. rossicum* plants showing recovery of inoculated and non-inoculated fungi from surface disinfected roots. n = total number of plants that were assessed for recovery of inoculated fungi.

Fungal treatment	n	Number of plants inoculated fungi recovered
M. phaseolina	6	6
Cadophora sp.	6	6
Ilyonectria radicicola	6	4
Ilyonectria sp.	6	4
Phaeocytostroma sp.	7	5
Phomopsis sp.	6	5
Colletotrichum sp.	6	1

Table S 4.3 Trial 2 number of plants showing recovery of inoculated and non-inoculated fungi from surface disinfected roots of *V. rossicum*, *A. syriaca*, and *S. canadensis*. n = total number of plants that were assessed for recovery of inoculated fungi.

Plant species	Fungal treatment	n	Number of plants inoculated fungi recovered
V. rossicum	Sterile control	3	NA
	Cadophora sp.	3	2
	I. radicicola	3	2
	M. phaseolina	3	3
A. syriaca	Sterile control	3	NA
	Cadophora sp.	3	3
	I. radicicola	3	2
	M. phaseolina	3	3
S. canadensis	Sterile control	3	NA
	Cadophora sp.	3	3
	I. radicicola	4	4
	M. phaseolina	3	3

Table S 4.4 Number of fungal morphotypes recovered from 20 surface-disinfected seeds of each of *V. rossicum*, *A. syriaca*, and *S. canadensis*.

Plant species	Number of morphotypes
V. rossicum	3
A. syriaca	4
S. canadensis	1

Chapter 5. Synthesis and Conclusions

This thesis comprehensively demonstrated that *V. rossicum* is able to form associations with a large number of taxa of root-associated fungi, including many fungi that are known pathogens on other plant species, but that it gains a net benefit from all soil biota. *Vinceoxicum rossicum*'s ability to associate with many AM and non-AM fungi may explain its ability to invade many habitats and become dominant. Importantly, I have been able to open the black box of root-associated fungal communities and tested some of their functions in terms of plant-growth responses. Overall, *V. rossicum* had higher biomass in the presence of soil biota compared to sterile soil in its invasive range, even when the substrate contained fungi that are known pathogens on other plant species (chapters 3 and 4). *Vincetoxicum rossicum* was rapidly colonised by many AM fungal taxa, and there was no evidence for convergence of root AM fungal community composition between uninvaded and invaded soil over one growth season (chapter 2). There were also no clear temporal patterns over decades of invasion for the entire root-associated fungal community, AM fungi or an accumulation of pathogens (chapter 3). In addition, *V. rossicum* associated with a large number of fungi that are known pathogens on other plant species which did not reduce its biomass (chapters 3 and 4). However, *V. rossicum* associates with many fungi, some of which were shown to reduce biomass of a commonly co-occurring native in its invasive range, *S. canadensis* (chapter 4). Together, these results indicate that *V. rossicum* is able to associate with a wide range of root-associated fungi, and gains a net benefit from soil biota in Ontario.

Overall, all experiments demonstrated that *V. rossicum* gains a net benefit from associating with soil micro-organisms because it consistently had higher biomass in the presence of soil biota compared to sterile soil (chapters 3 and 4). This was also true when the only microbe in the soil was a known pathogen on other plant species (chapter 4). These patterns indicate that *V. rossicum* could experience enhanced mutualisms, where an invasive species gains a relatively larger benefit from biota in its invasive range compared to its native range (Reinhart and Callaway, 2004); however, this would require comparing growth in soil from

its native range. It also questions whether high growth of *V. rossicum* is dependent on mycorrhiza *per se*, as suggested by Smith et al. (2008), especially since low AM fungal colonisation or high numbers of arbuscules did not reduce biomass (chapter 3). This suggests that low AM fungal colonisation may be offset by other soil biota, or that high AM fungal colonisation or arbuscules are not required for high biomass, at least in *V. rossicum* (chapter 3). Positive feedback with the AM fungal community was observed at one site (chapter 2) but may not be generalisable (chapter 3). This provides caution for conclusions from the many plant-soil feedback studies that are based use soil collected from only a small range.

One theory of invasion success is that plants can invade disturbed sites more easily than intact vegetation because resources such as soil nutrients are released (Davis et al., 2000; Shea and Chesson, 2002). However, *V. rossicum* is able to establish in disturbed areas as well as intact vegetation and it is plausible that this is due to its ability to rapidly associate with and gain benefit from soil biota. Perhaps it also gains benefits from intact vegetation through common mycelial networks that may transfer nutrients between plant species (Smith and Read, 2008; Weremijewicz and Janos, 2013; Fellbaum et al., 2014). For example, my most recently invaded site was Luther Marsh Wildlife Management area (chapter 3) and the invasion was represented by a small patch of *V. rossicum* plants that had not been observed prior to 2012. In fact, I could excavate all the plants when collecting. This site is an undisturbed pine forest that is popular with hunters, which is likely how *V. rossicum* was inadvertently introduced via seed. Combined with its ability to tolerate a wide range of climate conditions and vegetation types (DiTommaso et al., 2005a; Miller et al., 2007; Kricsfalusy and Miller, 2010; Sanderson and Antunes, 2013), the only factor that appears hinder range expansion by *V. rossicum* in Ontario is propagule pressure (Catford et al., 2009).

The two chapters that explicitly addressed temporal changes in root-associated fungal communities showed surprisingly different results than I had hypothesised based on previous work with other plants (chapters 2 and 3). I had expected to observe fine scale changes in composition of AM fungal communities over one growth season, but instead *V. rossicum* was rapidly colonised by many AM fungi and most of these stayed in the plant over 29 weeks (chapter 2). Since many plant-soil feedback studies often ‘train’ soil for much shorter periods of time than this, it is not unreasonable to have expected significant changes in the AM fungal communities on this time scale. However, when I used plants from multiple sites over 2-100

years of invasion there were also no temporal patterns in composition of AM fungi, fungi that are known pathogens on other plant species, or all root-associated fungi (chapter 3). In addition, soil used from different invasion ages did not show any differences in biomass in the glasshouse. Again, this highlights that *V. rossicum* rapidly forms associations with many root-associated fungi and gains a net benefit. A strong temporal pattern in root-associated fungi may be more easily observed in a plant species that is more dependent on plant-soil feedbacks for invasion success (Levine et al., 2006).

Chapter 2 showed that plants in invaded soils had more similar AM fungal composition than those in uninvaded soils, supporting the idea that *V. rossicum* may recruit particular fungal taxa over decades of invasion in two sites. However, chapter 3 showed that AM fungal composition did not differ in plants at sites of different invasion ages. Although these results appear to be contradictory, plants in chapter 3 were from many more sites and, importantly, they were over a much larger spatial scale. Therefore, the effect of invasion age may have been overridden by site- specific factors causing noise in any temporal pattern (Peay and Bruns, 2014; Kivlin et al., 2014). To truly test if the plant favours recruitment of a particular group of fungi and test convergence, the preferred taxa would need to be available in the soil and, ideally, the pool of fungal taxa available in the soil would be known. We might expect that sites in chapter 2 would have a more similar pool of AM fungi than those in chapter 3 because they were within 1 km of each other. Since plants in previously invaded soil were more similar in composition than those in uninvaded soil (chapter 2), this indicates that *V. rossicum* may have preferences for particular AM fungal taxa, even though it is obviously able to associate with a wider group of root-associated taxa, i.e., it is ‘bet-hedging’ (Lekberg and Koide, 2014). In addition, it effectively formed arbuscules for nutrient transfer in previously uninvaded soil (chapter 3), which may give it a competitive advantage over co-occurring plants that may be slower to form these associations, further supporting the enhanced mutualism hypothesis (Reinhart and Callaway, 2004). A proper test of the enhanced mutualisms hypothesis would require comparisons of colonisation rates of *V. rossicum* in soil from its invasive range in North America and its native Ukraine.

Although data from the invasion chronosequence over 100 years presented no support for the PAID hypothesis (Flory and Clay, 2013), I still consider this an important hypothesis that may be relevant for ecological communities and conservation management. Other studies have

shown that this phenomenon could be occurring in other plant invasions (Simberloff and Gibbons, 2004; Hawkes, 2007; Diez et al., 2010; Flory et al., 2011; Dostál et al., 2013); perhaps *V. rossicum* is an anomaly or a non-soil enemy will prove to be more important for *V. rossicum* decline. The observation of a stem pathogen on *V. rossicum* in New York state from a site that was only invaded 20 years ago implies that this is plausible, and also suggests two things: 1) time and pathogen accumulation may be less important than acquiring one important pathogen that severely reduces growth and fitness; and 2) as species expand their ranges they are more likely to encounter an effective enemy. The second point is intuitive because time and space in species invasions are bound to each other. The rate of range expansion could be a better predictor of pathogen accumulation than time since introduction, especially when we consider the time required for pathogens to evolve to colonise the novel host and grow to reach densities to disperse. It will be easier to understand the relative importance of each of these mechanisms in invasion dynamics as studies on pathogen accumulation become increasingly common.

Vincetoxicum rossicum associates with many fungi that are known pathogens on other plant species (chapters 3 and 4). Instead of reducing its growth, at least some of these fungi appear to directly increase its biomass, while simultaneously reducing biomass of a common co-occurring native plant, *Solidago canadensis* (chapter 4). It may be worthwhile testing the novel weapons hypothesis with *V. rossicum*, to investigate if soil biota in its invasive range show reduced pathogenicity on the invasive species because it is not pre-adapted (Callaway and Ridenour, 2004; Callaway et al., 2005). Testing this hypothesis fully would require pathogens from *V. rossicum*'s native range and perhaps including a range of plant genotypes from the native and invasive range that may differ in susceptibility. The observed results could also be a phylogenetic effect in the Apocynaceae because the pathogens also did not show a pathogenic effect on *A. syriaca*, but more replicate species within this family would be needed to test this. The fact that *V. rossicum* may act as a pathogen reservoir for *S. canadensis* to mediate competition via soil microbes is intriguing and requires further work. Since *S. canadensis* is commonly found with *V. rossicum* the relevance of these effects to their interaction in the field is unknown. Specifically, we need to know how common these pathogens are in *S. canadensis* and if it associates with them in the field, but more importantly, explicitly testing competitive interactions between *V. rossicum* and *S. canadensis* in the presence of these microbes.

Many of the results obtained in this thesis were not expected, based on previous theories and hypotheses. It is difficult to know how generalisable these results from this one species to other invasive plants, supporting the importance of gathering information on species that do not support prevailing hypotheses. In addition, the importance of soil biota for mediating or changing with invasive plants is increasingly recognised, but more studies are needed to open the black box and identify not only what is there, but how or even if they are affecting plant growth. As demonstrated, this can be done with traditional microbiology and plant pathology techniques (chapter 4). The next step is to understand the relative importance of the ability to associate with and gain benefit from a range of soil biota compared to other mechanisms of invasion (Suding et al., 2013).

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