100 YEARS Journal of Ecology

British Ecological Society

Journal of Ecology 2015, **103**, 1557–1569 doi: 10.1111/1365-2745.12459

Temporal dynamics of plant–soil feedback and root-associated fungal communities over 100 years of invasion by a non-native plant

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Summary

1. Pathogens can accumulate on invasive plants over time, which could lead to population declines. The time required for these dynamics to occur is unknown and seldom addressed. Furthermore, no study has assessed plant–soil feedback while characterizing plant pathogen and mutualist root fungal communities in the context of invasion time.

2. We used a plant–soil feedback study and 454 pyrosequencing to investigate pathogen accumulation over 100 years on a highly invasive plant in eastern North America that shows localized declines, *Vincetoxicum rossicum* (Apocynaceae).

3. We collected soil from five sites representing each of four invasion periods of *V. rossicum* across Ontario, Canada (old, *c.*100 years; intermediate, 50–60 years; young, < 12 years; and uninvaded), and grew *V. rossicum* in these soils in a glasshouse study. Our hypothesis was that plants grown in soils invaded for longer periods of time would experience less positive feedbacks compared to those grown in more recently invaded or uninvaded soils. We collected roots of *V. rossicum* from the invasion periods and performed 454 pyrosequencing targeting fungi. We hypothesized that the abundance and richness of fungi that are known plant pathogens would be higher in roots from older invasions compared to more recent invasions.

4. Contrasting with our hypothesis, *V. rossicum* experienced overall growth promotion due to soil biota, regardless of invasion period. *Vincetoxicum rossicum* roots were colonized by a large number of fungal taxa, including many known plant pathogens or mutualistic arbuscular mycorrhizal fungi. However, we found no evidence of pathogen accumulation in older invaded sites in terms of species composition, richness or abundance.

5. *Synthesis*. Our consistent results in the glasshouse and the field highlight the strength of combining high-throughput sequencing data with plant–soil feedback experiments. We showed that the roots of *Vincetoxicum rossicum* (Apocynaceae) were colonized by many fungal taxa, but found no evidence for changes in plant growth or accumulation of fungal pathogens with longer invasion time. High pathogen loads may not lead to concurrent declines in invasive plants. Plant invasions, as demonstrated by *V. rossicum*, may be unpredictable in their ability to accumulate pathogens capable of leading to population declines.

Key-words: 454 pyrosequencing, fungi, invasion ecology, pathogen accumulation, plant–soil feedback, soil, *Vincetoxicum rossicum*

Introduction

The enemy release hypothesis has been proposed as a key reason for the success of non-native invasive plants (hereafter invasive plants; Keane & Crawley 2002; Shea & Chesson 2002; Colautti *et al.* 2004). However, over time, pathogens may interact with invasive plants (Mitchell & Power 2003; Hawkes 2007) and this could eventually lead to declines in invasive plant populations (Diez *et al.* 2010; Flory & Clay 2013). High pathogen loads of non-native species are often positively correlated with residence time, but the time required for their effects to manifest in individuals or populations is unclear (Hawkes 2007; Mitchell *et al.* 2010). In addition, while invasive plant–pathogen interactions have been

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reported, concurrent declines in growth of invaders are seldom observed (Simberloff & Gibbons 2004; Hawkes 2007; Strayer 2012). This could, in part, be due to the evolution of increased competitive ability (Blossey & Notsold 1995), phenotypic plasticity (Richards *et al.* 2006; Lamarque *et al.* 2015), novel chemical defences (Callaway & Ridenour 2004) or positive effects of mutualisms that may counteract the negative effects of pathogens (Borowicz 2001; Reinhart & Callaway 2006; Morris *et al.* 2007). For example, arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) within the Glomeraceae family are particularly effective at protecting plants from root-associated pathogens (Maherali & Klironomos 2007; Sikes, Cottenie & Klironomos 2009). Studies investigating ideas of pathogen accumulation in invasive plants have rarely considered invasion time and have neglected to characterize the pathogen and mutualist communities that underlie plant growth responses.

Plant soil feedback is defined as the action exerted by a plant on soil properties (biota in the case of the present study) that, in turn, results in changes that are reflected in responses by this same plant (van der Putten *et al.* 2013). Invasive plants commonly experience positive plant–soil feedback (increased growth in their own soil) in their invaded ranges but negative feedback (reduced growth in their own soil) in their native range. This pattern is thought to be due primarily to enemy release, although interactions with mutualisms may also play a role (Reinhart & Callaway 2006; Callaway *et al.* 2011; Gundale *et al.* 2014; but see Beckstead & Parker 2003). However, negative plant–soil feedbacks may accrue with time, which is congruent with pathogen accumulation theory (van der Putten, van Dijk & Peters 1993; van de Voorde, van der Putten & Bezemer 2012; Flory & Clay 2013). Using non-native plants that had been established in New Zealand for over 210 years, Diez *et al.* (2010) showed that species with longer residence times experienced stronger growth reductions compared to those more recently established. A similar pattern was shown for *Heracleum mantegazzianum* in the Czech Republic, where biomass of this plant was lower in soils collected from sites invaded for at least 48 years compared to more recently invaded soils (Dostál *et al.* 2013). However, neither of these studies characterized associated changes in soil biota and, consequently, it is assumed that pathogens are driving these patterns. Since interactions between plants and soil fungi range along a continuum from pathogenic to mutualistic (Agrios 2005; Smith & Read 2008), the fungal Kingdom can be considered suitable for linking temporal dynamics of plant–soil feedbacks with pathogen accumulation in invasive species, information that is currently lacking (Flory & Clay 2013).

Here, we assessed the accumulation of below-ground fungal pathogens by investigating links between plant growth and root-associated fungi through invasion time. We used *Vincetoxicum rossicum* as our study species, a highly invasive plant in parts of North America including southern Ontario in Canada and some eastern states in the USA (Sheeley & Raynal 1996; Cappuccino, Mackay & Eisner 2002). We chose this highly invasive species because it is a perennial with a dense root system and its roots are colonized by fungal pathogens and AM fungi in North America (Couture, Brisson & Émond 2003; Bongard *et al.* 2013). In addition, a fungal stem pathogen, *Sclerotium rolfsii* VrNY, has recently been shown to be causing population declines at a site in New York state in the USA (Gibson 2012; Gibson *et al.* 2014), and there is anecdotal evidence for localized population declines in Ottawa, Ontario, Canada, where it has been present since the 1950s (McNeill 1981; Fletcher Wildlife Garden 2014). First, we identified 20 sites with similar environmental and soil conditions that had been invaded by *V. rossicum* ranging in time from zero to *c.* 100 years. We then conducted a glasshouse experiment using soils from these sites to test the hypothesis that plant–soil feedback would become more negative in soils where *V. rossicum* had been established for longer periods of time. In addition, we used high-throughput sequencing (454 pyrosequencing) to characterize root-associated fungal taxa in *V. rossicum* collected from the sites representing different invasion periods. We expected roots from older sites to have higher species richness and abundance of potential pathogens (defined here as fungi that have been recorded as pathogens on any plant species), compared to roots from more recently invaded sites. Since *V. rossicum* readily associates with AM fungi (Smith *et al.* 2008; Bongard *et al.* 2013), we did not expect differences in AM fungal species richness with invasion time. However, we expected AM fungal community composition to change towards taxa associated with pathogen protection, specifically those within the Glomeraceae (Maherali & Klironomos 2007; Sikes, Cottenie & Klironomos 2009).

Materials and Methods

STUDY SPECIES AND SITE SELECTION

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 & Raynal 1996; Cappuccino, Mackay & Eisner 2002). Originally from the Ukraine and southwest Russia (Pobedimova 1952 in DiTommaso, Lawlor & Darbyshire 2005), there is evidence that *V. rossicum* experiences release from above-ground enemies in Ontario (Agrawal *et al.* 2005). In North America, *V. rossicum* can establish under a wide range of light, moisture and climate conditions as well as in many different soil and vegetation types (DiTommaso, Lawlor & Darbyshire 2005; Averill *et al.* 2011; Sanderson, Day & Antunes 2015). The first record of *Vincetoxicum* spp. in Ontario was in Toronto in 1889 (Moore 1959). It was subsequently reported to be abundant in the Don Valley in Toronto in the early 20th century (Scott 1913) and was widespread throughout southern Ontario by the 1970s (Pringle 1973). *Vincetoxicum rossicum* is now considered a noxious weed and is a primary target for invasive species management in this region (Miller *et al.* 2007; Anderson 2012).

We determined the approximate dates of establishment of *V. rossicum* in 15 sites throughout southern Ontario using a combination of literature, herbarium records and accounts from local land

managers and conservation authorities (Table S1, Supporting information). We located five sites for each of three invasion periods: old (first record of *V. rossicum c.* 100 years ago), intermediate (50–60 years) and young (< 12 years; Fig. 1 and Table S1). We also located five uninvaded sites that had no record of ever being invaded by *V. rossicum* but consisted of suitable habitats for this species as they were similar to those invaded (Cappuccino 2004; DiTommaso, Lawlor & Darbyshire 2005; Miller *et al.* 2007; Anderson 2012). Collections of plant material and soil from the invaded sites were made within dense *V. rossicum* stands, except for the more recently invaded sites (i.e. young invasion period) where there were relatively fewer *V. rossicum* plants (see Table S1 and Fig. S3 for a list sampling locations, including the information source, description of vegetation type and photographs). Concentrations of ammonium, nitrate, phosphorus and pH of soil from the uninvaded sites were within the same ranges as those of soil from the invaded sites (Fig. S1). All sites, including those within the City of Toronto, were in naturalized areas and had experienced minimal or no management for invasive species eradication (C. Webster, City of Toronto, personal communication with NJD).

PLANT GROWTH RESPONSES

To test the functional effects of soil microbial communities in response to *V. rossicum* invasion, we conducted an experiment in the glasshouse using soil collected from each of the 20 sites of the four invasion periods (old, intermediate, young, uninvaded; Fig. 1, Table S1). At each site, soil and root material were collected directly from under *V. rossicum* plants within an area of *c.*3 m^2 and to a depth of *c.*30 cm and placed in an opaque plastic bag then refrigerated at 4 °C. Spades were thoroughly scrubbed with undiluted household bleach and rinsed with water between sites. Soil from each site was homogenized through a 4-mm sieve. Approximately 18 L of soil were sieved from each site; 9 L were kept as live inoculum and 9 L kept for sterilization. All equipment was soaked in a diluted bleach solution for a minimum of 20 min and all benches were bleached and rinsed between processing soil from each site. Since roots are important sources of inoculum for some root fungi (Tommerup & Abbot 1981; Klironomos & Hart 2002; Agrios 2005), we accounted for this by collecting roots while sieving and cutting them into 2- to 3-cm pieces using flame-sterilized scissors. Then, 25 g of roots was mixed into the live soil and another 25 g was mixed into the soil for sterilization, which standardized organic matter between live and sterile soil. Roots in the soil from invaded sites were comprised entirely of *V. rossicum*, while roots from the uninvaded sites consisted of a mixture of the plants present in the soil. The soil for sterilization was autoclaved (121 °C and 18 psi for 90 min), stored at room temperature for one day and re-autoclaved. Sterilized soil was stored at room temperature for 10 days to allow for mineralization. Live soil was stored at 4 °C to minimize potential changes in microbial communities.

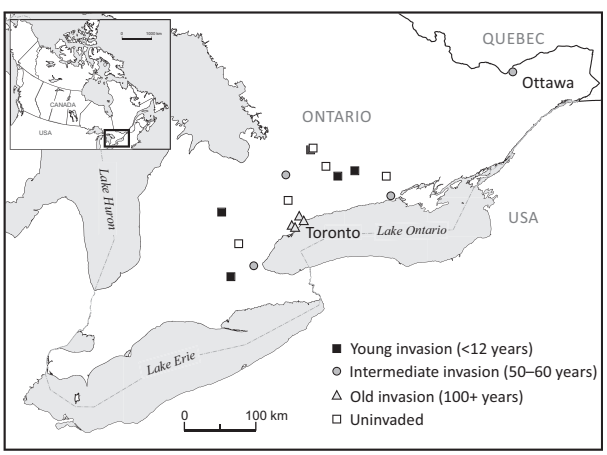

Fig. 1. Map of 20 sites representing different periods of *Vincetoxicum rossicum* invasion in Ontario, Canada (Table S1).

We established seven replicate pots for each of the 20 sites, with an additional seven sterile controls, making a total of 147 pots, each 2.8 L. Each pot contained a substrate mixture comprised of equal volumes of homogenized sterile sand (non-calcareous 'B' sand, Hutcheson Sand and Mixes, Huntsville, Ontario, 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 mixture of sterile soil from all 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. This was done to minimize differences in soil nutrients, texture and organic matter (Bever 1994; Pendergast, Burke & Carson 2013). Control pots contained a 1400-mL mixture of sterilized soil from all sites. A 1-cm layer of sterile sand was placed on the surface of all pots to prevent cross-contamination. Pots were lined with mesh and placed on saucers to prevent substrate and nutrient loss.

Seeds collected from one site in Toronto, Ontario, were stratified at 4 °C between moist sheets of filter paper for 3 weeks prior to planting. Five weeks prior to potting the experiment, seeds were surface-disinfected for 3 min in 70% ethanol followed by 10 min in 1% sodium hypochlorite and three rinses in sterile water. They were then pre-germinated in a tray with an equal mixture of sterile sand, turface and soil (autoclaved twice at 121 °C and 19 psi for 90 min). One seedling was planted into each pot using bleach-disinfected equipment. Plants that did not survive the transplant were replaced within 1 week (11 of 147 plants). Additional plants that grew in the soil were clipped at the surface to ensure there was only one *V. rossicum* plant per pot. Plants were watered with reverse osmosis water between 2 and 7 days each week over the course of the experiment and were grown under a 14-h/10-h day/night photoperiod with temperature ranging between 20 and 24 °C. Pots were re-randomized monthly. A low concentration of slow release fertilizer was added to each pot 5 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 of growth. At this time, plants ranged in height from 7.8 to 93.4 cm and many had formed seed pods (see Results). Plants in 133 pots survived the course of the experiment. Our final data set consisted of 31 plants for each of the intermediate and old invasion periods, 32 plants for the uninvaded and young invasion periods, and seven controls; mortalities were therefore not distributed in any pattern related to invasion period. No plants died in the sterile controls. At harvest, shoots were separated and roots were thoroughly washed in tap water, dried with a paper towel and weighed. Two random subsamples of roots were stored in tissue cassettes in 70% ethanol to estimate fungal colonization. The roots were re-weighed after subsamples were taken to enable back-calculation of total root biomass after drying. Shoots and roots were stored in separate paper bags and dried at 60 °C for at least 3 days before weighing. Dry weights were measured twice to ensure consistency and accuracy.

Colonization by AM and non-AM fungi was assessed in three randomly selected replicates per site and three controls. Roots were

cleared in 10% KOH, rinsed and placed in a 5% ink–vinegar solution for 10 min at 90 °C (Pelikan black ink; Vierheilig *et al.* 1998). Destaining was achieved by placing the roots in 50% glycerol for a minimum of 3 h before mounting onto slides. Per cent colonization of roots by fungi was measured using the grid intersection technique for 100 intersections at 200× magnification (McGonigle *et al.* 1990). Presence of AM fungal hyphae, arbuscules, vesicles and non-AM fungal hyphae was recorded at each intersection; non-AM fungi could be pathogens. Slides were assigned randomized numbers to avoid potential treatment bias when assessing colonization.

ROOT-ASSOCIATED FUNGAL COMMUNITIES

In the second part of this study, we characterized the root-associated fungal communities of *V. rossicum* using four plants from each of five sites for each of the old, intermediate and young invasion periods. At each site, four dense clusters of *V. rossicum* plants within 5 m were excavated. This ensured that the root samples were from different crowns, but minimized spatial differences in terms of microsites and soil conditions. Plants were excavated *c.* 30 cm deep and placed in plastic bags with the rhizosphere and bulk soil intact. Plants were refrigerated at 4 °C for up to 5 days. Roots attached to crowns of similar sizes were randomly subsampled, one from each plant cluster, and washed in tap water. Roots were patted dry with paper towels, cut into 2-cm pieces and surface-disinfected in 70% ethanol for 3 min, followed by 10 min in 1% sodium hypochlorite, and three rinses in sterile water, then patted dry with sterile paper towels. We surface-disinfected the roots to ensure that only fungi capable of colonizing *V. rossicum* would be captured. We confirmed that no fungal growth occurred on acidified potato dextrose agar swiped with a surface-disinfected root. Two 100 mg samples were taken for each plant and stored in separate sterile 2-mL microcentrifuge tubes at −20 °C until DNA was extracted.

For DNA extraction, frozen roots were homogenized by placing four sterilized 3-mm tungsten carbide beads into each microcentrifuge tube with the roots, immersing in liquid nitrogen for 30 s and shaking at 30 mHz for 1 min 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 homogenization with care to avoid thawing roots. DNA was extracted with the Macherey–Nagel Nucleospin Mini kit using CTAB cell lysis buffer (Macherey–Nagel and Co. KG, Düren, Germany). We sterilized beads between extractions by washing with deionized water, soaking in UltraClean Lab Cleaner to remove nucleases and nucleic acids (MoBio Laboratories, Carlsbad, CA, USA), rinsing thoroughly and autoclaving (121 °C and 18 psi for 15 min). DNA from two technical replicates of each plant was pooled, purified and stored at −20°C. DNA from two plants from one site of the old invaded period was lost due to a labelling error, resulting in DNA from roots of 58 plants.

Purified DNA was sent to McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada) for 454 library preparation and sequencing using fungal-specific primers ITS1F (CTTGGTCATTTAGAGGAAGTAA; Gardes & 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 amplified fungal DNA, and the second PCR was required to add the extended primers with 454 adapters and molecular identification (MID) tags (Lindahl *et al.* 2013). 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 10× 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 min, followed by 35 cycles of 95 °C for 20 s, annealing at 60 °C for 30 s, elongation at 72 °C for 45 s, followed by a final elongation step at 72 °C for 5 min. 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 min, followed by 15 cycles at 95°C for 15 s, annealing at 68°C for 30 s, elongation at 72°C for 60 s, followed by a final elongation step at 72°C for 3 min. DNA concentrations were measured by picogreen and standardized to 50 ng per sample prior to sequencing on a Roche 454 GS FLX+ sequencer.

SEQUENCE ANALYSIS AND DESIGNATION OF OTUS

Bioinformatics processing of 454 sequences was performed by McGill University and Génome Québec Innovation Centre (Montréal, Québec, 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 nucleotides with quality scores lower than 15 were discarded. Remaining reads were denoized by clustered at 100% similarity, followed by clustering at 99% similarity in DNACLUST v.3 (Ghodsi, Liu & Pop 2011). 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, Liu & Pop 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. The resulting data set was not randomly subsampled ('rarefied') prior to statistical analyses because this has been shown to be problematic, particularly in terms of inflating variability and increasing the probability of type II errors (McMurdie & Holmes 2014). Instead, rare OTUs that occurred in only one of the 58 samples were removed to further denoize the data, which is a common practice in community analysis (McCune & Grace 2002; Legendre & Legendre 2012). Sequence data were submitted to GenBank (Accession Numbers: KP867137 - KP867614).

Categorizing OTUs as potential pathogens and AM fungi

To investigate whether there was greater pathogen accumulation on *V. rossicum* at older compared to more recently invaded sites, all

OTUs identified to genus were classified according to whether or not they could be a plant pathogen. A potential pathogen on *V. rossicum* was defined as an OTU in a genus that contained at least one species identified as a plant pathogen on any plant species in the literature. This coarse categorization was required because few studies have explicitly tested pathogens on *V. rossicum* or its close relatives (Berner *et al.* 2011; Gibson 2012; Gibson *et al.* 2014). We searched for occurrences of potential pathogens in two ways. Initially, we searched the fourth edition of the *Names of Plant Diseases in Canada* book (Couture, Brisson & Émond 2003). For genera that were not recorded in this book, we searched the Web of Science data base in July 2014 using the following search terms for each genus: 'genus name' AND pathog*. Given the importance, widespread abundance and the fact that they comprise a Phylum that is primarily mutualistic (Smith & Read 2008; Hoeksema *et al.* 2010; Treseder 2013), we focussed on AM fungi to investigate changes in mutualistic fungi. All OTUs in the Phylum Glomeromycota were considered to be beneficial for plant growth (Schussler, Schwarzott & Walker 2001; Smith & Read 2008; www.mycobank.org).

STATISTICAL ANALYSES

All statistical analyses were performed in R version 3.1.0 (R Core Development Team 2014) using packages where specified.

Plant growth responses

To test for plant growth effects of *V. rossicum* in relation to invasion period, we fitted generalized linear mixed models with invasion period as a fixed effect and either total biomass or root–shoot ratio as the response. Pre-planned reverse Helmert contrasts were specified to assess responses in different invasion periods. Contrast 1: sterile control compared to the mean of live soil combined (all invasion periods including uninvaded), which assessed *V. rossicum* growth when growing with and without soil biota. Contrast 2: uninvaded compared to the mean of young, intermediate and old invasion periods combined, which assessed *V. rossicum* growth in invaded and uninvaded soils. Contrast 3: young compared to the mean of the intermediate and old invasion periods combined, which assessed *V. rossicum* growth in recently invaded soils compared to those where it had been established for 50–100 years. Contrast 4: intermediate compared to old invasion period, which assessed *V. rossicum* growth in soils where it had been established for 50–60 years compared to soils where it had been established for *c.* 100 years. We fitted these models using function 'lme' in package nlme (Pinheiro *et al.* 2014). We fitted a generalized linear mixed model with the same fixed effect and pre-planned contrasts and the number of flowers and seed pods per plant as the response. This model was fitted with a Poisson response using 'glmmPQL' in package MASS to account for overdispersion (ratio of variance to mean greater than 1; Venables & Ripley 2002; Crawley 2013). We included site as a random effect in all models. Results for all models were unchanged when fitted with invasion period as a continuous variable (results not shown), so we only present results with invasion period as a categorical predictor and the pre-planned contrasts to be able to include comparisons with the sterile controls.

To test for differences in fungal colonization of *V. rossicum* roots in each invasion period, we fitted generalized linear mixed models with invasion period as a fixed effect and AM fungi (including hyphae, arbuscules and vesicles), arbuscules only, vesicles only or non-AM fungi as the response. These models were run with colonization as a binomial response for proportion data. Since there was no colonization by AM or non-AM fungi in sterile controls, we omitted these samples and specified pre-planned reverse Helmert contrasts. Contrast 1: uninvaded compared to the mean of young, intermediate and old invasion periods combined, which assessed colonization in invaded and uninvaded soils. Contrast 2: young compared to the mean of the intermediate and old invasion periods combined, which assessed colonization in recently invaded soils compared to those where it had been established for 50–100 years. Contrast 3: intermediate compared to old invasion period, which assessed colonization in soils where *V. rossicum* had been established for 50–60 years compared to soils where it had been established for *c.* 100 years. We fitted these models using 'glmmPQL' in package MASS to account for overdispersion of the binomial distribution (Venables & Ripley 2002; Crawley 2013). We included site as a random effect in all models. Results for all models were unchanged when fitted with invasion period as a continuous variable (results not shown). We adjusted *P*-values using the false discovery rate (FDR) method due to multiple tests on the same data using function 'p.adjust' in base R (Benjamini & Hochberg 1995; Waite & Campbell 2006).

Root-associated fungal communities

We displayed the OTU composition of the root-associated fungi for all 58 plants using a principal coordinate analysis (PCoA). The PCoA was fitted in 6 dimensions using function 'cmdscale' in base R. The Bray–Curtis dissimilarity was applied to OTU abundances, in terms of number of reads. These dissimilarities were further used in a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) to test for significant predictors of root-associated fungal community composition. Predictors were invasion period, 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 of all the soil variables (data not shown). We specified site as a random effect and *P*-values were estimated from 1000 randomizations using the 'adonis' function in VEGAN 2.0-10 (Oksanen *et al.* 2013). Due to the uncertainty surrounding the accuracy of abundance data from 454 pyrosequencing (Amend, Seifert & Bruns 2010), we also fitted the PCoA and PERMANOVA using the presence absence data and the results were unchanged (results not shown).

To investigate changes in potential pathogen community composition, we repeated the analyses that had been used for the entire root-associated fungal community for the potential pathogens. In addition, we fitted generalized linear mixed models to assess potential pathogen accumulation using both the richness (number of potential pathogen OTUs, Poisson response) and the abundance of pathogens (proportion of reads that were potential pathogens, binomial response). In each model, the fixed effect was invasion period and pre-planned reverse Helmert contrasts were specified. Contrast 1: young compared to the mean of the intermediate and old invasion periods combined, which assessed pathogens in recently invaded soils compared to those where *V. rossicum* had been established for 50–100 years. Contrast 2: intermediate compared to old invasion period, which assessed pathogens in soils where *V. rossicum* had been established for 50–60 years compared to soils where it had been established for *c.* 100 years. Models were fitted using function 'glmmPQL' in package MASS to account for overdispersion (Venables & Ripley 2002; Crawley 2013). We specified site as a random effect in each model. Analyses for investigating AM fungal composition, richness and abundance were the same as for the potential pathogen analysis. Results for all models were unchanged when models were run with invasion period as a

continuous variable (results not shown). We adjusted *P*-values using the FDR method due to multiple tests on the same data using function 'p.adjust' in base R (Benjamini & Hochberg 1995; Waite & Campbell 2006).

Results

PLANT GROWTH RESPONSES

Vincetoxicum rossicum had higher total biomass, lower root–shoot ratios and a higher number of flowers and seeds in live soil from all invasion periods compared to the sterile controls (Fig. 2, Tables S2 and S3). This was true even for soil collected from uninvaded sites where *V. rossicum* had not 'trained' the soil (Fig. 2, Tables S2 and S3). Invasion period did not have a significant effect on total plant biomass, root–shoot ratio or the number of flowers and seed pods (Fig. 2, Tables S2 and S3). A high percentage of root intersections were colonized by AM fungi in soil from all invasion periods (range: 0.21–0.91; Fig. 3). Plants growing in soils from the young invasion period had significantly lower AM fungal root colonization than those growing in the intermediate and old invasion periods (Fig. 3; Table S4). Plants in uninvaded soil had significantly higher root colonization by arbuscules compared to those from the young, intermediate and old invasion periods (Fig. 3; Table S4).

ROOT-ASSOCIATED FUNGAL COMMUNITIES

All the sequences detected in the roots of 58 plants were fungal sequences, which confirms the validity of the chosen primers. The final data set comprised 406 684 reads clustered into 478 OTUs at 97% similarity occurring in at least two of the 58 plants. This was an adequate sampling effort to capture the community (Fig. 4). Each plant had a mean of 7012 reads (range: 2299–11 995) and 49 OTUs (range: 7–68). 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 plants, and only three OTUs occurred in more than 35 plants: an isolate in the genus *Cyanonectria* was present in 52 plants, an isolate of *Plectosphaerella* in 55 plants and an isolate of *Phycomyces* in 57 plants. Four fungal phyla were detected in total: 238 OTUs belonged to the Glomeromycota (100 280 reads), 183 to Ascomycota (292 236 reads), 15 to Basidiomycota (7052 reads) and eight to Zygomycota (5148 reads). The remaining 34 OTUs (1968 reads) could not be assigned to phyla based on the RDP classification with a confidence >0.5. A total of 23 fungal Orders and 48 Families were identified, although 68 and 86 OTUs could not be assigned to Order or Family, respectively (Tables S5 and S6).

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 period (Fig. 5). A group of samples clustered in the top right of the PCoA plot were characterized by abundant OTU 238153 and OTU 131560 (both isolates of Helotiaceae), OTU 32872 (an isolate of *Plectosphaerella*) and OTU 225843 (an isolate of *Paraglomus*), but these were not related to particular sites or invasion periods. This was supported by the PERMANOVA, where invasion period and soil parameters (ammonium, nitrate, phosphorus and pH) were not significant predictors of root-associated fungal composition (Table 1). However, there were qualitative differences between invasion periods at the Order level. Orders Cantharellales and Coniochaetales were only present in the young invasion period, and Sordariales was only present in the intermediate and old invasion periods (Fig. S2). Glomerales was represented by a large number of OTUs in all invasion periods (Fig. S2).

Potential pathogen and mutualist communities

Of the 478 unique OTUs, 351 were identified to genus that represented 62 fungal genera (i.e. each genus was represented by multiple species at 97% similarity; Table S7). The literature search resulted in 32 genera being classified as potential pathogens (Table S7). However, invasion period and the soil parameters were not significant predictors of potential pathogen composition (Table 1). The generalized linear mixed models showed no evidence for greater richness or abundance of potential pathogens on

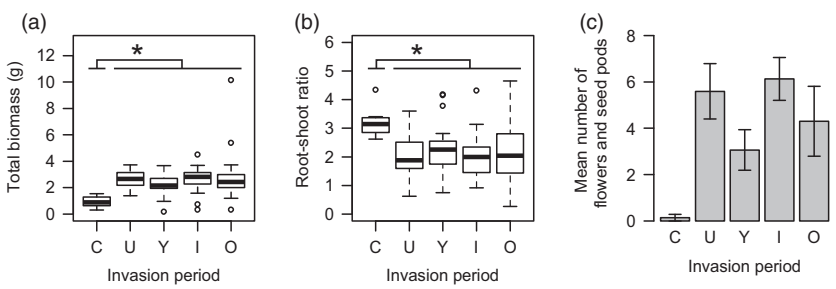

Fig. 2. *Vincetoxicum rossicum* plant growth responses measured as total dry biomass (g) (a), root–shoot ratio (b) and the number of flowers and seed pods (c) from 133 plants from 20 sites corresponding to four invasion periods and sterile controls. C = sterile control, U = uninvaded, Y=young invasion (< 12 years), I = intermediate invasion (50-60 years) and O = old invasion (*c.*100 years). On (a) and (b), the box represents the interquartile range, the band represents the median, and 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 S2). On (c), error bars are standard errors of the mean.

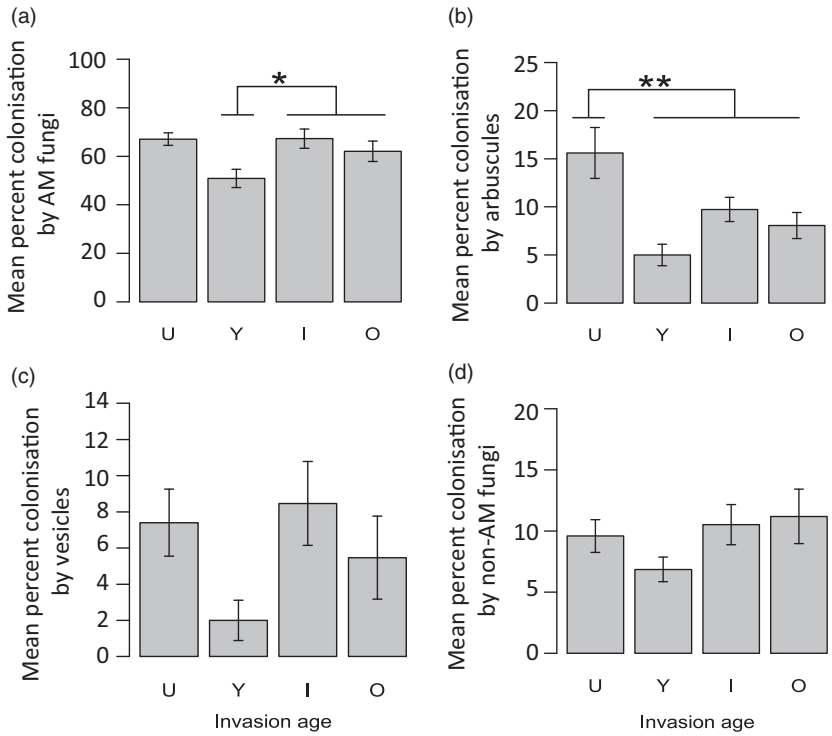

Fig. 3. Mean per cent colonization of *Vincetoxicum rossicum* colonized by AM fungi (i.e. hyphae arbuscules and vesicles combined) (a), arbuscules only (b), vesicles only (c) and non-AM fungi (d) for 60 plants from 20 sites corresponding to four invasion periods. U = uninvaded, Y = young invasion (< 12 years), I = intermediate invasion (50-60 years) and O = old invasion (*c.*100 years). 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 S4). Sterile controls showed no colonization and are not shown.

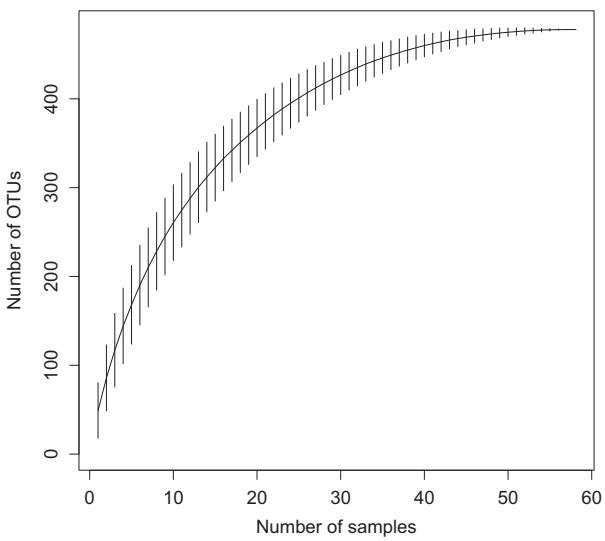

Fig. 4. Species accumulation curve showing mean and standard deviation based on 1000 permutations of 454 pyrosequencing results of 478 fungal OTUs identified from roots of 58 *Vincetoxicum rossicum* plants from 15 sites in three invasion periods.

V. rossicum at older compared to more recently invaded sites (Fig. 6, Table S8).

A total of 238 OTUs in eight genera were within Phylum Glomeromycota and used in the analysis of AM fungal communities. Only one plant from the young invasion period lacked AM fungal OTUs. Invasion period and the soil parameters were also not significant predictors of AM fungal composition (Table 1). In addition, plants from different invasion periods did not differ in richness or abundances of AM fungi (Fig. 6, Table S9).

Discussion

In this study, we searched for evidence of accumulation of below-ground potential pathogens, changes in AM fungal communities and changes in plant–soil feedback through invasion time. Contrasting with our first hypothesis, the glasshouse experiment showed that *V. rossicum* experienced overall increased growth in live soil compared to sterile soil and growth was not reduced when grown in soils with longer invasion histories. Further work with 454 pyrosequencing on roots from sites invaded by *V. rossicum* for different periods of time showed no consistent changes in root-associated fungal communities. In addition, there were no changes in community composition, species richness or abundance of either AM fungi or fungi that had been recorded as pathogens on other plant species.

GROWTH RESPONSES TO SOIL BIOTA

In contrast with other studies investigating plant growth responses in relation to invasion time (Diez *et al.* 2010; Dostál *et al.* 2013), we found no evidence for reduced growth (i.e. negative plant–soil feedback) in soil from longer periods of *V. rossicum* invasion (Fig. 2). Instead, *V. rossicum* experienced increased growth in live soil compared to sterile soil and gained a net benefit from soil biota in its invasive range (Fig. 2). This suggests that, at least in Ontario, *V. rossicum* invasion may not be hindered by accumulation of below-ground pathogens, including non-fungal soilborne pathogens that we did not explicitly assess in the 454 pyrosequencing. Observed localized population declines of *V. rossicum* in North America (Gibson 2012; Fletcher Wildlife Garden 2014;

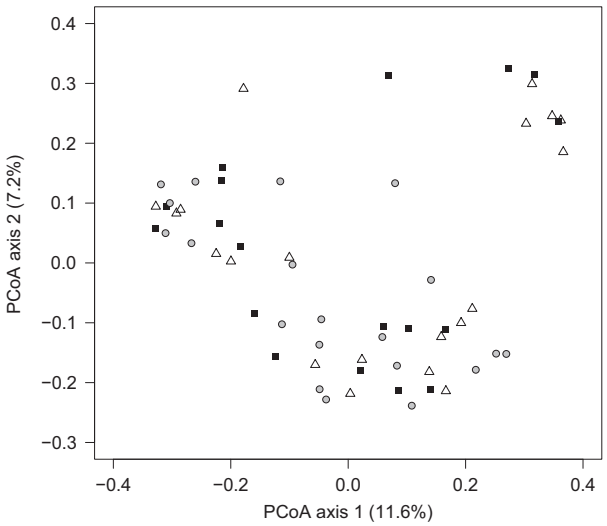

Fig. 5. Site scores of principal coordinate analysis (PCoA) using Bray–Curtis dissimilarity on abundance data for 478 fungal OTUs identified from roots of 58 *Vincetoxicum rossicum* plants from 15 sites in three invasion periods. White triangles represent young invasion (< 12 years), grey circles represent intermediate invasion (50-60 years) and black squares represent old invasion period (*c.*100 years). Values in parentheses on axes correspond to the amount of variation explained by each axis (total variation explained = 36.4%).

Gibson *et al.* 2014) may not be related to pathogen accumulation over time. For example, a fungal strain of *S. rolfsii* causes wilting and mortality at one site in New York state (Gibson 2012; Gibson *et al.* 2014), but *V. rossicum* was not observed at that site until after 1995 (C. Southby & L. R. Milbrath personal communication with NJD). This pathogen has not been observed in Ontario and was not detected in our fungal DNA sequences. These results highlight the importance of regional-scale studies when investigating long-term invasion dynamics.

Results obtained from field-collected roots using 454 pyrosequencing are consistent with those of the glasshouse experiment in that they did not support our hypothesis of potential pathogen accumulation with longer invasion time (Fig. 6). While soil fungi are an appropriate indicator of below-ground enemies, it is possible that *V. rossicum* has accumulated above-ground enemies over time (e.g. foliar pathogens, insect herbivores). In addition, patterns of pathogen accumulation may emerge as *V. rossicum* continues to invade and expand its range in North America; data from Hawkes (2007) and Diez *et al.* (2010) suggest that *c.* 200 years may be required and *V. rossicum* was first recorded in Ontario 126 years ago. However, Flory, Kleczewski & Clay (2011) showed growth reductions in *Microstegium vimineum* due to pathogen accumulation after just 100 years and Dostál *et al.* (2013) showed growth reductions in *Heracleum mantegazzianum* after just 50 years of invasion. Since enemies in plant–soil feedback studies are seldom identified, observations of stronger negative feedbacks over time could be due to factors other than pathogen accumulation. For example, Seifert, Bever & Maron (2009) found that *Hypericum perforatum* had reduced mycorrhizal dependency in its invasive range compared to its native range, which may change as a function of time.

Our finding that *V. rossicum* exhibited increased growth in the presence of soil biota but no growth reductions in relation to invasion period, even in the presence of many potential pathogens, demonstrates the strength of combining plant growth response data with characterization of root-associated fungal communities. Many invasive plants have higher biomass in live soil from their invaded range compared to sterile soil and show the opposite response in soil from their native

Model	Variable	Variation explained (%)	d.f.	SS	MS	Pseudo *F*	*P*
Root-associated fungi	Invasion period	3.72	2	0.70	0.35	1.08	0.708
	Soil ammonium	1.83	1	0.34	0.34	1.07	0.518
	Soil nitrate	2.49	1	0.47	0.47	1.45	0.725
	Soil phosphorus	1.56	1	0.29	0.29	0.91	0.725
	Soil pH	2.97	1	0.56	0.56	1.73	0.518
	Residuals	87.43	51	16.49	0.32		
	Total	100.00	57	18.86			
Potential pathogens	Invasion period	3.78	2	0.54	0.27	1.09	0.450
	Soil ammonium	1.30	1	0.19	0.18	0.75	0.725
	Soil nitrate	1.61	1	0.23	0.23	0.93	0.450
	Soil phosphorus	1.43	1	0.20	0.20	0.83	0.708
	Soil pH	3.81	1	0.54	0.54	2.21	0.450
	Residuals	88.07	51	12.57	0.25		
	Total	100.00	57	14.27			
AM fungi	Invasion period	3.33	2	0.83	0.42	0.94	0.708
	Soil ammonium	2.18	1	0.54	0.54	1.23	0.518
	Soil nitrate	2.06	1	0.52	0.52	1.16	0.725
	Soil phosphorus	1.67	1	0.42	0.42	0.94	0.725
	Soil pH	1.98	1	0.49	0.49	1.11	0.708
	Residuals	88.78	50	22.23	0.42		
	Total	100.00	56	25.04			

Table 1. Permutational analysis of variance (PERMANOVA) testing potential predictors of root-associated fungal, potential pathogens and AM fungal community composition based on the Bray–Curtis dissimilarity on abundances from 454 pyrosequencing of the roots of 58 plants of *V. rossicum* from 15 sites corresponding to three invasion periods: young (< 12 years), intermediate (50–60 years) and old (*c.*100 years). *P*-values were adjusted to account for multiple tests on the same data.

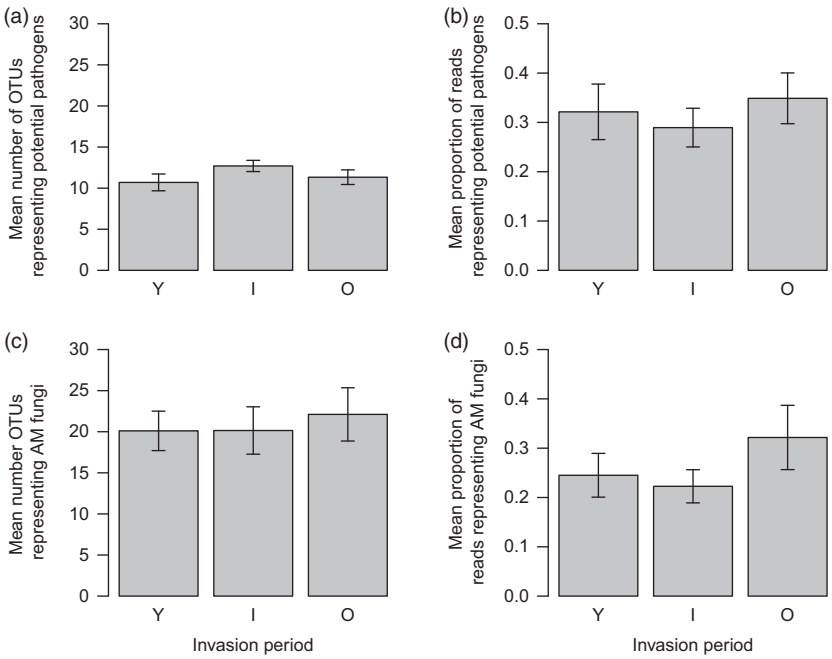

Fig. 6. Groups of root-associated fungi in *Vincetoxicum rossicum* showing mean number of OTUs of potential pathogens (a), mean proportion of reads of potential pathogens (b), mean number of AM fungal OTUs (c) and mean proportion of AM fungal reads (d) from the roots of 58 plants from 15 sites of different invasion periods. Y=young invasion (< 12 years), I=intermediate invasion (50-60 years) and O=old invasion (*c.*100 years). A potential pathogen was defined as an OTU in a genus that contained at least one species identified as a plant pathogen on any plant species (Table S7).

range (Reinhart *et al.* 2003; Callaway *et al.* 2004; Reinhart & Callaway 2006; Gundale *et al.* 2014; Maron *et al.* 2014). Differentiating whether this is due to accumulation of mutualists (Reinhart & Callaway 2006) or a lack of enemies is challenging (Colautti *et al.* 2004), particularly in the case of *V. rossicum* because it associates heavily with both mutualists and pathogens (Bongard *et al.* 2013; Table S7). However, the high colonization rates of AM fungi on *V. rossicum* in the glasshouse experiment (Fig. 3), as well as many OTUs and sequence reads of AM fungi in field-collected roots (Fig. 6), particularly those in the Glomeraceae (Table S6; see below), could offset negative effects of potential enemies on *V. rossicum* (Borowicz 2001; Maherali & Klironomos 2007; Sikes, Cottenie & Klironomos 2009). In addition, plants growing in the uninvaded soil in the glasshouse experiment had significantly higher colonization of arbuscules compared to the young, intermediate and old invasion periods (Fig. 3). This shows that *V. rossicum* can rapidly form associations with AM fungi during initial establishment and access soil nutrients via these structures (Smith & Read 2008), which could be an important mechanism underlying its invasibility (Pringle *et al.* 2009). However, since the observed differences in the colonization of AM fungi and arbuscules did not affect biomass as would be expected (Fig. 3; Treseder 2013), this suggests that other soil biota that we did not characterize could also be important in mediating plant growth in this species.

We cannot exclude the possibility that the absence of effects observed in the glasshouse experiment was due to an artefact of the study design. For example, our space-for-time substitution approach to invasion periods means that abiotic and some biotic conditions may have varied with sampling site. However, the soil parameters evaluated were comparable across sites (Fig. S1). Furthermore, Dostál *et al.* (2013) found significant changes in plant growth over invasion time using a similar approach, giving us no reason to consider the study design to be the cause of non-significant results. It is possible that we inadvertently selected for a particular group of soil microbes by mixing soil with an artificial substrate and placing it under glasshouse conditions, potentially influencing plant growth responses (e.g. Sýkorová *et al.* 2007). However, we minimized this possibility by including field-collected roots in the potted substrate, which can serve as an important propagule source for root-associated fungi (Tommerup & Abbot 1981; Klironomos & Hart 2002; Agrios 2005). If pathogenic fungi were present in these roots, then it is reasonable to assume that they could have infected the emerging seedlings, including those pathogens that are facultative saprobes (Agrios 2005). In addition, results from the glasshouse experiment and the 454 pyrosequencing were consistent in supporting the idea that pathogen accumulation has not occurred below-ground in *V. rossicum* at these sites. Our lack of significant plant–soil feedback effects in relation to invasion time with this plant is important to gain a better understanding of plant invasions (Strayer 2012) as well as from the perspective that there can be a bias towards the publication of significant results (Møller & Jennions 2001).

The lack of pathogenic symptoms in the glasshouse experiment, even in the presence of many potential pathogens, could be due to plant age, environmental conditions or the lack of an intermediate host (Jarosz & Davelos 1995; Agrios 2005; Álvarez-Loayza *et al.* 2011). High pathogen loads may not always be reflected in plant growth (Torchin & Mitchell 2004), and pathogen load may be less important than pathogen specificity because one particular isolate can cause dramatic effects on individual plants or populations (e.g. Flory, Kleczewski & Clay 2011). We recognize that our method of categorizing fungal genera as potential pathogens based on other plant species was very coarse because fungal pathogens are known to be host or even genotype specific (Jarosz & Davelos 1995; Agrios

2005). However, we used this method because few studies have tested for pathogenic effects of fungi on *V. rossicum* (Berner *et al.* 2011; Gibson 2012; Gibson *et al.* 2014) and none have explicitly tested root pathogens. Further tests have shown that *V. rossicum* can experience neutral or positive growth effects from fungi that are known pathogens on other plants and reduce growth of co-occurring plant species (Day, Dunfield & Antunes unpublished data). This demonstrates that *V. rossicum* may be symptomless to fungal pathogen infection and highlights the limitations of generalizing about the ecological function of fungi solely based on rDNA sequencing. Nevertheless, if fungal groups commonly associated with pathogenicity had increased with invasion time, our combinatorial approach would have detected this. Future research should take steps towards developing methodologies capable of combining community level identification with pathogenicity-associated markers.

Another possibility is that pathogenic taxa able to infect *V. rossicum* were present but not in the pathogenic strain at the appropriate stage of the life cycle. For instance, the anamorph fungal forms (asexual) are more likely to cause disease symptoms than the teleomorph (sexual; Agrios 2005), and until recently, these morphs have been assigned different names because they are morphologically distinct (Alexopoulos, Mims & Blackwell 1996; Domsch, Gams & Anderson 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 plant (Shenoy, Jeewon & Hyde 2007; Hawksworth 2011). These difficulties highlight the need to combine high-throughput sequence data with glasshouse experiments and traditional plant pathogenicity testing to be able to infer function (van der Putten *et al.* 2007).

THE FUNGAL COMMUNITY ASSOCIATING WITH ROOTS OF *VINCETOXICUM ROSSICUM*

This study contributes to the growing body of knowledge from high-throughput sequencing data describing fungal communities of plants (Öpik *et al.* 2009; Bass & Richards 2011; Lekberg *et al.* 2012). We found that the roots of *V. rossicum* are able to associate with a broad range of fungal taxa: 478 fungal OTUs were identified across 58 plants (Tables S5-S7). Most fungal taxa in this study are new records on this plant and only four of the 62 OTUs that were identified to genus have prior records on *V. rossicum*: *Colletotrichum* (Berner *et al.* 2011), *Fusarium*, *Glomus* and *Plectosphaerella* (Bongard *et al.* 2013). We found that *Glomus*, *Rhizophagus* and *Plectosphaerella* were highly abundant, in terms of both number of OTUs and number of sequence reads (Table S7). The abundance of *Glomus* and *Rhizophagus* supports previous evidence that *V. rossicum* is highly mycorrhizal (Smith *et al.* 2008; Bongard & Fulthorpe 2013). In addition, these are in the Glomeraceae family, which are thought to have better pathogen-protecting functions compared to other families within the Glomeromycota (Maherali & Klironomos 2007; Sikes, Cottenie & Klironomos 2009). 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 shorter in Ascomycota, which may have led to preferential amplification of this group (Bellemain *et al.* 2010).

Our data showed no significant relationship between invasion period and root-associated fungal community composition (Table 1, Fig. 5). There were some observable differences in Orders between invasion periods (Fig. S2). For example, Cantharellales and Coniochaetales were only present in the young invasion period and Sordariales was only present in the intermediate and old invasion periods (Fig. S2). However, these Orders were only represented by one or two OTUs in the entire data set and overall representation of all Orders in different invasion periods was similar (Table S5, Fig. S2). A group of samples clustered in the PCoA plot were characterized by abundant OTU 238153 and OTU 131560 (both isolates of Helotiaceae), OTU 32872 (an isolate of *Plectosphaerella*) and OTU 225843 (an isolate of *Paraglomus*), but these were not restricted to particular sites, soil parameters or invasion periods (Fig. 5). High within-site variation in root-associated fungal communities from high-throughput sequencing appears to be common (Lekberg *et al.* 2012; Blaalid *et al.* 2014; Peay & Bruns 2014). This could be because soil parameters that are important for root and soil fungal communities, such as pH (Stotzky 1997; Lekberg *et al.* 2007) and phosphorus (Jakobsen, Abbott & Robson 1992; Dickson, Smith & Smith 1999; Kiers *et al.* 2011), can vary over fine spatial scales and may also explain why our site-level measurements of these variables were not significant predictors of composition (Table 1). Surrounding plant species composition, plant age and localized environmental factors are also known to be important determinants of root and soil fungal community composition (Hausmann & Hawkes 2009; Hart *et al.* 2014; Peay & Bruns 2014). These patterns highlight the importance of testing hypotheses of soil microbial community structure using multiple plant species, soil types and sites. Regardless of these potential confounding effects, had fungal communities significantly differed both in composition and if *V. rossicum* feedbacks had changed due to time since invasion, our combination of testing for soil biota feedbacks and characterizing fungal communities in field-collected roots should have been able to detect it.

Vincetoxicum rossicum produces a secondary compound that appears to be unique to North America, (-)-antofine, and has been shown to have antifungal properties (Cappuccino & 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 & Ridenour 2004; Callaway, Hierro & Thorpe 2005; Cappuccino & Arnason 2006). However, the present study and others have demonstrated that *V. rossicum* associates with a diversity of taxa across the fungal Kingdom (e.g. Berner *et al.* 2011; Gibson 2012; Bongard *et al.* 2013), which does not support the idea that (-)-antofine is an important mechanism of invasion for *V. rossicum*. It is possible that the fungi that

colonize *V. rossicum* are resistant to (-)-antofine, and we speculate that this may act as a filter, thereby explaining the lack of significant temporal changes in fungal composition. Further work would be required to test this hypothesis.

Conclusions

This study supports growing evidence suggesting that *V. rossicum* is able to associate with a broad range of root-associated fungi (Pringle *et al.* 2009; Bongard *et al.* 2013). Although 454 pyrosequencing showed that *V. rossicum* roots were colonized by many fungi that are known pathogens in other plant species, there was no accumulation of these fungi in roots from sites with longer invasion time. In addition, the glasshouse study demonstrated that this plant experienced overall increased growth in the presence of soil biota regardless of time since invasion. As such, our study suggests that some invasive plants may not accumulate pathogens predictably with time for at least over a century after establishment. These results call for more studies to characterize soil biota and feedbacks across multiple spatiotemporal scales and a range of sites for multiple plant invaders.

Acknowledgements

We thank Carleton University, Couchiching Conservancy, the Grand River Conservation Authority, Kawartha Conservation, Lake Simcoe Conservation Authority, Trent University, Toronto Parks and Rouge Park for granting us permission to collect soil and plants. Thanks to L. Sanderson for assisting with plant and soil collection and performing the soil nutrient analyses and G. J. Boland for insightful discussions about fungal pathogens. Sincere thanks to Génome Québec and particularly J. Trembley for performing the 454 pyrosequencing and bioinformatics. We thank Y. Lekberg and E. Wandrag and two anonymous referees for providing thoughtful comments that improved this manuscript. U. Kim, F. Small, M. Mucci and T. Slimmon assisted in the glasshouse and helped with sample processing. Funding was provided by the Natural Sciences and Engineering Research Council of Canada, Ontario Ministry of Natural Resources, New Zealand Federation of Graduate Women Fellowship and the University of Guelph International Graduate Scholarship.

Data Accessibility

- Sequence data submitted to GenBank (Accession Numbers: KP867137 - KP867614
- Fungal colonization, Pathogen table, Plant dry weights and site by species matrix from 454 pyrosequencing: Dryad Digital Repository doi:10.5061/dryad.vp530 (Day, Dunfield & Antunes 2015)

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Received 15 April 2015; accepted 20 July 2015
Handling Editor: Peter Thrall

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Soil parameters for the 20 field sites.

Figure S2. Mean number of OTUs of each fungal Order identified from 454 pyrosequencing.

Figure S3. Photos of the sampling sites described in Table S1.

Table S1. Sample locations and site descriptions.

Table S2. Statistical analysis - glasshouse experiment: total plant biomass; root-shoot ratio.

Table S3. Statistical analysis - glasshouse experiment: number of flowers and seed pods.

Table S4. Statistical analysis for root colonization by AM fungi.

Table S5. Distribution of OTUs based on 454 pyrosequencing: Orders.

Table S6. Distribution of OTUs based on 454 pyrosequencing: Families.

Table S7. Distribution of OTUs based on 454 pyrosequencing: Genera.

Table S8. Statistical analysis – field collected roots - for potential fungal pathogens.

Table S9. Statistical analysis – field collected roots - for AM fungi.