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Original article

Changes in arbuscular mycorrhizal fungal communities during invasion by an exotic invasive plant

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ABSTRACT

Exotic invasive plants can show strong plant–soil feedback responses, but little is known about time scales for significant changes in soil microbial communities to occur after invasion. Previous work has suggested that plant invasions can modify arbuscular mycorrhizal (AM) fungal community structure. However, there is a lack of understanding about how long it takes for these changes to develop. To test this we investigated temporal changes in AM fungal communities colonising the invasive plant *Vincetoxicum rossicum* (Apocynaceae). We hypothesised that AM fungal community structure would change in a particular direction during the invasion process. We collected soil from two sites with a long history of invasion by this plant, with each site having paired invaded and uninvaded plots. Soil from these plots was used in a glasshouse experiment to characterise AM fungal community structure in the roots of *V. rossicum* at different times throughout a simulated growing season. AM fungal community structure differed between invaded and uninvaded plots. However, contrasting with our hypothesis, AM fungal communities colonising *V. rossicum* growing in soil from uninvaded plots did not change towards those in plants growing in previously invaded soil. Our data suggest that changes to AM fungal communities in the presence of *V. rossicum* require longer than the first growing season after establishment to develop.

1. Introduction

Studies on plant–soil feedback, where the soil microbial community changes in response to a particular plant species and subsequently affect plant growth, show that exotic invasive plants often benefit from positive feedback (van der Putten et al., 2007a; Pendergast et al., 2013; Bardgett and van der Putten, 2014). It has been hypothesised that soil mutualisms drive this positive feedback (Klironomos, 2002; Zhang et al., 2010; Bever et al., 2012). However, little is known about the time scale required for significant changes to occur in mutualistic soil microbial communities after establishment of an exotic plant invader. Hawkes et al. (2013) recently showed that the direction of plant–soil feedbacks can change over relatively short periods of time. This indicates that investigating the temporal dynamics of key soil microbial communities is important to understand invasiveness and environmental impacts of exotic plant invaders.

Arbuscular mycorrhizal (AM) fungi (Phylum Glomeromycota) establish obligate mutualisms with most land plants, where plants provide the fungal partners 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 fungal community structure is determined by a range of biotic and abiotic factors, including plant host (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). Large inter- and intra-annual variability in AM fungal communities has also been observed in field studies (Husband et al., 2002; Liu et al., 2009; Dumbrell et al., 2011; Sánchez-Castro et al., 2012; Helgason et al., 2014). AM plants are colonised by multiple fungal species and species isolates, some

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more beneficial than others (van der Heijden et al., 1998; Eom et al., 2000; Lewandowski et al., 2013). In addition, selective recruitment between particular plant species and AM fungal isolates has been demonstrated (Bever et al., 2009; Kiers et al., 2011; Fellbaum et al., 2014), which may contribute to positive feedback (Bever et al., 2012).

Exotic invasive plants (hereafter invasive plants) can depend 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). One theory is that highly mycorrhizal and successful invasive plants may be less selective and associate with a wide range of AM fungi across locations (van der Putten et al., 2007b; Pringle et al., 2009; Nuñez and Dickie, 2014). However, AM fungal communities can change in the presence of invasive plants (Zhang et al., 2010; Pendergast et al., 2013). For example, *Centaurea maculosa* imposed significant changes to AM fungal communities in Montana grasslands (Mummey and Rillig, 2006). Little is known about how quickly AM fungal communities change in response to invasive plants. This knowledge may contribute to better understand feedbacks, invasiveness, and impacts of plant invasions (Levine et al., 2006; Kardol et al., 2013).

Vincetoxicum rossicum (Kleopow) Barbar. (Apocynaceae) (syn. *Cynanchum rossicum* (Kleopow) Borhidi; dog-strangling vine) is a highly invasive perennial 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., 2005), in North America *V. rossicum* can establish under a range of light, moisture, and climate conditions, as well as in many different soil and vegetation types (DiTommaso et al., 2005; Averill et al., 2011; Sanderson and Antunes, 2013; Sanderson et al., 2015). *V. rossicum* is highly mycorrhizal dependent and readily associates with many AM fungal species in its invaded range (Smith et al., 2008; Bongard et al., 2013).

The main objective of this study was to determine whether AM fungal community structure changes in response to *V. rossicum*. We collected soil from two field sites with paired plots that had either no record of invasion or decades of invasion by this species in Canada. This soil was used in a glasshouse experiment to monitor changes in AM fungal communities colonising *V. rossicum* roots over the course of 29 weeks (i.e., equivalent to one growing season). We hypothesised that at the onset of the experiment AM fungal community structure colonising plants growing in soils from invaded and uninvaded field plots would be different. However, if AM fungal communities change in response to *V. rossicum*, then we expected that the AM fungal community structure in plants growing in the uninvaded soil would become more similar to those in the invaded soil over time. In addition, we measured plant growth responses throughout the experiment. We expected that general soil biotic effects of *V. rossicum* invasion would have growth effects on this plant species in soils with different invasion histories.

2. Materials and methods

2.1. Soil and seed collection

Soil was collected from Toronto Zoo, ON, Canada (N 43°49′7″, W −79°11′8″) at each of two sites approximately 1 km apart (hereafter referred to as site 1 and 2). Given that *V. rossicum* invasion in this area was homogeneous, two sites were considered to be sufficient to assess patterns in AM fungal communities. Within sites, soil was collected from two paired plots: one with no record of *V. rossicum* invasion ('uninvaded') and the other with a dense population of *V. rossicum* ('invaded'). *V. rossicum* had been present for at least 20 years in the invaded plots, which were last mown or managed in the early 1990s (J. Bell, Toronto Zoo Manager, personal communication). Therefore, these plots are considered to have been trained by *V. rossicum* for multiple decades. The two sites were chosen within a small geographic area to minimise environmental and soil differences. Since the sites had similar management histories, it is an assumed that plant communities in the uninvaded plots are representative of those present prior to invasion by *V. rossicum* and that all locations were at the same successional stage (see Table S1). Approximately 60 L of soil was collected from each plot from the top 20–30 cm, covering an area of approximately 3 m^2. Soil was homogenised by sieving (4 mm) and stored in air tight, opaque containers for transport back to the laboratory where they were kept at 4 °C until the start of the experiment four days later. On site, all containers, spades and soil sieves were scrubbed and soaked in diluted bleach for at least 20 min to prevent cross-contamination between plots. A subsample of soil from each site indicated that both sites had the same soil type: a Till Plain slightly alkaline (mean pH 7.93 ± 0.05) fine sandy loam. Soil fertility was similar among all four plots (Table S2).

Seeds of *V. rossicum* were collected five weeks prior to soil collection from opened seedpods within the invaded plots. Pappi were removed and seeds were stored in paper envelopes at 4 °C for approximately two weeks. Seeds were stratified between sheets of moist filter paper in the dark at 4 °C for 18 days (Smith et al., 2008). Prior to planting, seeds were surface disinfected in 10% bleach for three minutes and rinsed in sterile water.

2.2. Experimental design

The experiment was conducted in a glasshouse and each site was considered separately to assess reproducibility of patterns across sites. We used a completely randomised factorial design with two crossed factors: 'invasion' (soil with two categories: invaded and uninvaded) and 'time' (with 5 categories: harvests 1, 2, 3, 4, and 5, corresponding to 9, 13, 19, 24, and 29 weeks after planting) with four replicates per invasion–time combination. Four sterile control pots were also prepared for each plot, consisting of autoclaved soil (90 min at 121 °C and 18 psi), making a total of 8 controls and 48 experimental units per site. All potting equipment was disinfected by soaking in diluted bleach for at least 20 min. For each site, soil was sieved and thoroughly 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 a 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. 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 24 °C. Pots were randomised monthly. At week 11 (between harvests 1 and 2) 200 mg of 20-2-20 (Plant Products, ON, Canada) fertiliser was added to each pot (Plant Products, Brampton, ON, Canada).

2.3. 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). All controls were harvested at harvest 5. Equipment was disinfected between each replicate. Harvested plants were stored at 4 °C for no longer than two days prior to processing. Roots were thoroughly washed free of soil in cold tap water, patted dry with a clean paper towel, and weighed. A subsample of approximately 100 mg of roots was selected randomly, cut into 1–2 cm pieces, placed in sterile 2 ml microcentrifuge tubes, and frozen at −80 °C for subsequent DNA extraction. 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 calculated. All plants were weighed twice to ensure consistency and accuracy in dry weights.

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 s 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 with care to avoid the roots from thawing. 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 121 °C and 18 psi for 15 min. DNA was stored at −20 °C.

2.4. Characterisation of AM fungal communities

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 5× 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 95 °C for 3 min, 57 °C for 30 s and elongation at 72 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 30 s and elongation at 72 °C for 1 min. The last cycle was followed by a 5 min elongation at 72 °C. 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. The 1:999 dilution was used because 1:9 and 1:99 dilutions resulted in smeared or multiple bands. 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). These primers and the LSU region have been well-established for AM fungi (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 controls.

Four DGGE gels were run, one for each plot, each containing four replicates of each of the five harvest times for a total of 80 samples. Gels contained 8% acrylamide with a gradient of 20–50% denaturant and run in a D-Code system (BioRad, Hercules, CA, USA). 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 min) then voltage was increased to 75 mV and run for 17 h. Gels were stained with 0.02 SYBR green for 15 min, 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).

All bands, sequenced and unsequenced, were used to measure AM fungal richness and composition using pictures of the four DGGE gels and importing them into GeneTools (Syngene, Cambridge, UK). Each band was considered to be one isolate of AM fungi. Gels were aligned using internal standards based on multiple preliminary gels. The presence and absence of each band in each experimental unit (gel lane) was determined. AM fungal band richness was calculated by summing the number of bands in each lane. Presence/absence of bands was used instead of using DGGE band fluorescence intensity as a measure of abundance due to inconsistencies in band fluorescence between PCR products (Nakatsu, 2007). We considered DGGE an appropriate method to answer our questions about temporal changes in AM fungal communities because we did not need to know the identities of each isolate in each plant.

DNA sequences from bands were aligned and a phylogenetic tree was drawn using MEGA version 5.2 (Tamura et al., 2011) following methods 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 (Hall, 2000). Tree reliability was assessed by 2000 bootstrap replications and *Scutellospora* sp. was used as outgroup to root the tree (Hall, 2000) as it is in a different Order than all other sequences obtained.

2.5. 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. First, exploratory analysis of AM fungal band composition was conducted for both sites, invasion, and time categories by using principal co-ordinates analysis (PCoA) with 6 dimensions using function ‘cmdscale’ in base R with Jaccard's distance (McCune and Grace, 2002; Legendre and Legendre, 2012). Overall, we expected AM fungal community composition in the roots of *V. rossicum* to be less variable in soils

from the invaded plots than in those uninvaded. This was tested by a multivariate test of homogenous variances (Anderson, 2006) using the 'betadisper' function with Jaccard's distance. 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., 2013).

Second, for each site separately we investigated differences in AM fungal richness between invasion categories over time. 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), with an interaction term between invasion and time. To investigate the significance of invasion and time on band composition, a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) specifying Jaccard's distance was performed, with an interaction term between invasion and time. *P*-values were estimated from 1000 randomisations using the 'adonis' function in vegan package version 2.0-7 (Oksanen et al., 2013).

To investigate total biomass and root–shoot ratio responses, two generalised linear models were run for each site separately. The Gaussian link was specified for both models for continuous data (Crawley, 2002). Models were performed using function 'glm', specifying an interaction between invasion and time. Only data for 60 plants at harvests 2, 3, 4 and 5 were included in these models because all the root material from samples from harvest 1 and for four plants from harvest 2 were used for DNA extractions (two from each invasion category).

3. Results

3.1. Exploratory analysis

A total of 44 unique bands were distinguished across all 80 experimental units (lanes). There were between seven and 18 AM fungal bands per plant, with a mean of 12 ± 0.3 bands each. All of the 25 bands that were sequenced corresponded to AM fungi in the Order Glomerales (Fig. 1, Table S3).

The PCoA with all bands suggested that AM fungal composition differed between sites and invasion categories (Fig. 2). Most bands were present in both sites, indicating that any possible differences between sites in the PCoA may be 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 plants growing in invaded soil, while nine bands were unique to those growing in uninvaded soil across both sites. The multivariate test of homogeneity of variances between invasion categories showed that overall, AM fungal composition in plants in the invaded soils was more similar (i.e., there was less variation) than that in the uninvaded soils ($F_{1,38} = 17.74$, $P < 0.001$, 999 permutations; Fig. 2).

3.2. Did invasion by V. rossicum rapidly alter AM fungal community structure?

Consistently, there was no significant difference in AM fungal band richness between invaded categories over time at either site (Table S4, Fig. S1). For each site individually, the PERMANOVAs showed that invasion explained a significant proportion of variation in AM fungal composition (Table 1). At site 1, AM fungal composition changed significantly over time, and there was a significant interaction between invasion and time; AM fungal composition diverged between invaded and uninvaded soils at harvest 5. The invasion by time interaction was marginally significant for site 2 ($P = 0.059$); AM fungal composition diverged between invaded and uninvaded soils at harvests 2 and 5 (Table 1).

3.3. Did V. rossicum invasion experience feedback?

Plants growing in invaded soils consistently had higher total biomass than those in uninvaded soils, but this was only marginally statistically supported for site 2 (Table 2, Fig. 3). We found a significant interaction between invasion and time in site 2. After harvest 2, the total biomass of plants growing in invaded soil was consistently larger than that of plants growing in uninvaded soil (Table 2, Fig. 3). At each site, plants growing in invaded soil had lower root–shoot ratios than those in uninvaded soil. There was also a significant interaction between invasion and time for root–shoot ratio at both sites (Table 2). The root–shoot ratio of plants growing in invaded soil remained relatively constant over time, whereas that of plants growing in uninvaded soil kept increasing after harvest 3 (Table 2, Fig. 3).

4. Discussion

The objective of this study was to determine temporal dynamics of AM fungal community structure in response to *V. rossicum* invasion. Our results supported the hypothesis that AM fungal communities colonising plants growing in invaded and uninvaded soils were different (Table 1, Fig. 2). However, contrary to our expectation, AM fungal community structure in the uninvaded soils did not become similar to those in the invaded soils over the 29 weeks of the experiment (Table 1, Fig. 2). As hypothesised, we found contrasting plant growth responses between the two invasion categories, indicating that despite the potential filtering that the pot experiment in the glasshouse may have imposed, general soil biotic effects resulting from *V. rossicum* invasion were still captured (Table 2, Fig. 3).

We found that overall AM fungal community structure was different between plants growing in invaded and uninvaded soils collected from both sites (Table 1, Fig. 2). The PERMANOVAs showed that invasion explained a substantial proportion of the variation in AM fungal community structure; 23% and 25% in sites 1 and 2 respectively (Table 1). In addition, the homogeneity of variances test across both sites showed that AM fungal composition in plants in the invaded treatment was more similar than those in the uninvaded treatment. This indicates that *V. rossicum* invasion may have led to a particular AM fungal community over decades of invasion, which is consistent with other invasive plants (Mummey and Rillig, 2006; Zhang et al., 2010; Pendergast et al., 2013). It is possible that even longer time may be required for the AM fungal community composition in invaded areas to completely overlap with each other, as there is separation between the invaded soils on the PCoA (Fig. 2). Alternatively, there may never be a strong overlap in invaded communities, for example, if *V. rossicum* is an AM fungal generalist or if AM fungi are dispersal limited. It has been 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). Although AM fungal communities present at the invaded plots might have been shaped by environmental variables other than *V. rossicum* invasion, these plots were separated by over one kilometre and were heavily invaded by dense populations, suggesting that *V. rossicum* probably played a role in the observed similarities in AM fungal community structure (Table S1). What our results do show is that *V. rossicum* is able to invade and associate with many different AM fungal isolates.

V. rossicum was colonised by many AM fungal isolates by harvest 1 (Fig. S1). Over time, we expected that AM fungal community structure in plants growing in the uninvaded soil would become more similar to that of AM fungi colonising plants growing in invaded soils. However, instead, we observed AM fungal

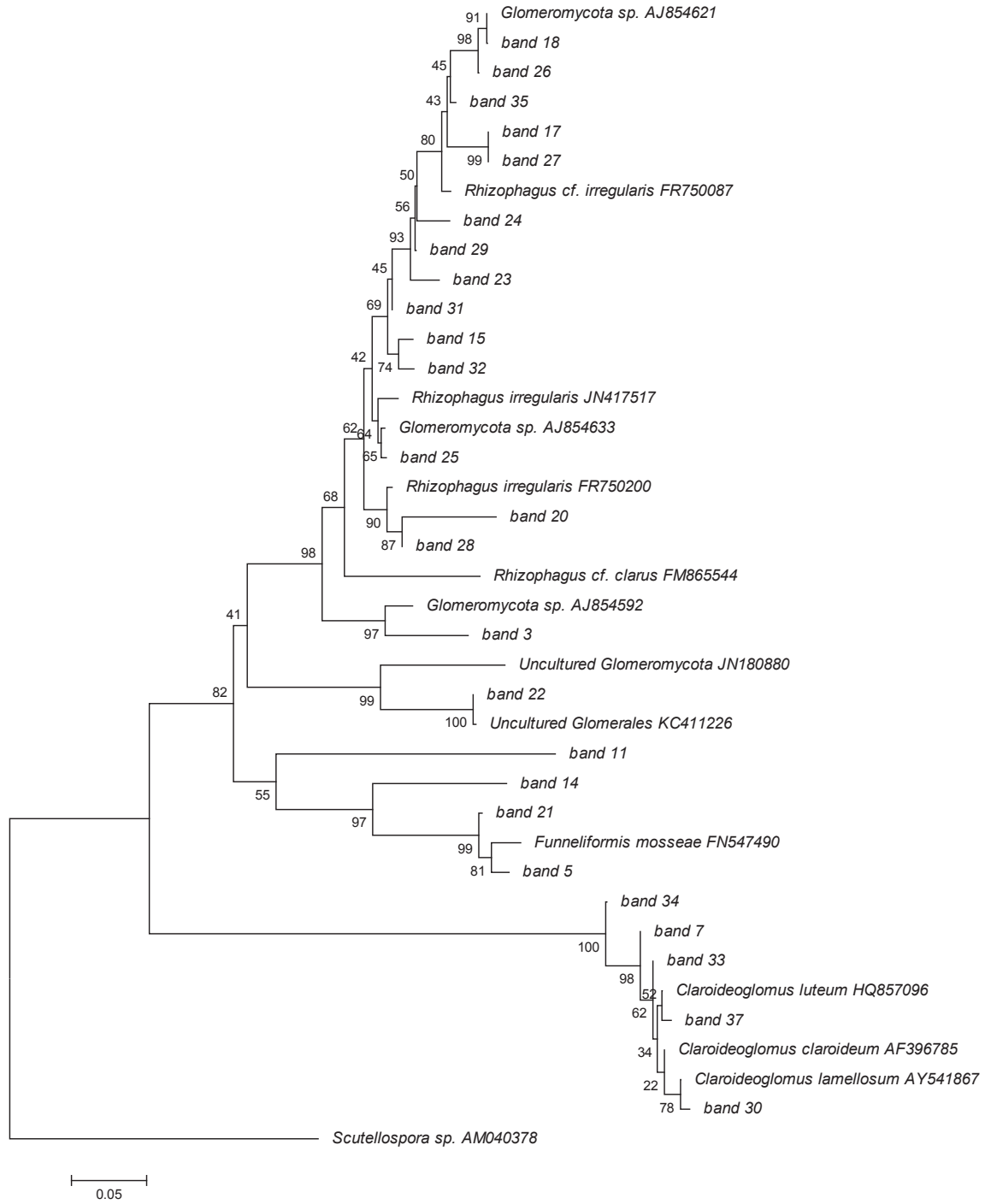

Fig. 1. Neighbour-Joining phylogenetic tree showing evolutionary relationships between sequenced DGGE bands and Genbank sequences. The optimal tree with the sum of branch length = 2.017 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 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. (AM040376) in the Order Diversisporales was used to root the tree.

communities changed without a clear direction (Fig. 2). That is, even though we detected a significant interaction between invasion and time in both sites, AM fungal communities did not become more similar over time, at least over the course of 29 weeks. It is unclear how long it may take for plant-induced changes in microbial communities to occur across plant species and edaphic conditions (Kardol et al., 2013). For *V. rossicum* we found that decades of invasion resulted in changes in AM fungal community structure but our data suggests these changes did not accrue over the course of one growing season. In contrast, Zhang et al. (2010) found that AM fungal communities changed in response to *Solidago canadensis* in as little as two growing seasons. It is possible that fine scale temporal patterns may have been clearer if abundances of each AM fungal isolate had been estimated from DGGE band brightness

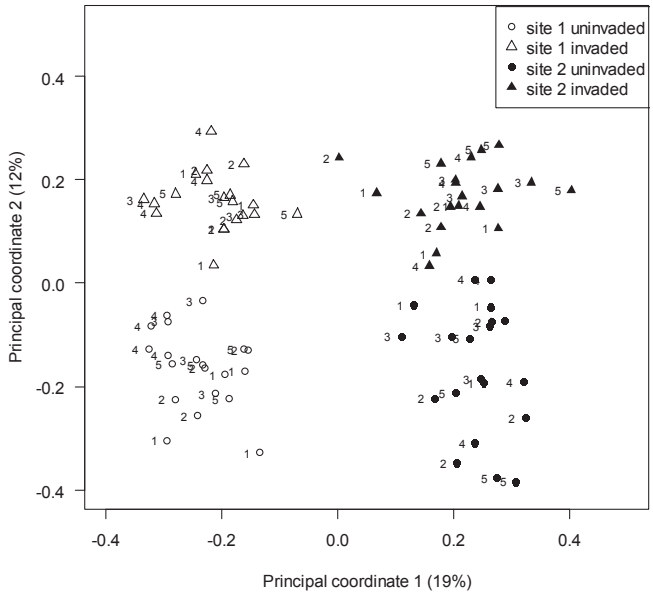

Fig. 2. Site scores of principal co-ordinates analysis (PCoA) using Jaccard's distance on AM fungal bands (n = 44) from denaturing gradient gel electrophoresis (DGGE), showing sites and invasion categories across all harvest times. Values in brackets on axes correspond to the amount of variation explained by each axis. Numbers next to points show harvest time.

Table 1
Results from permutational analysis of variance (PERMANOVA) for each site with Jaccard's distance on AM fungal composition based on DGGE bands.

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

(Merryweather and Fitter, 1998; Dumbrell et al., 2011). However, there is still much debate about the ability of any molecular method to estimate the relative abundances of fungal isolates in natural communities (Lindahl et al., 2013). It is further possible that if we had observed a strong temporal direction in AM fungal composition, then this could have been driven by succession of those fungi selected for by the glasshouse environment. Future studies addressing questions of how root-associated fungi change in the presence of an invasive plant could be done using experimental field studies or investigating sites that have been invaded for different periods of time.

Table 2
Results from generalised linear models for total biomass and root–shoot ratio from harvests 2 to 5 for each site (all root mass for harvest 1 was used for molecular work). Values in brackets are standard errors.

Predictor	Total biomass			Root–shoot ratio		
	Coefficient	*t*	*P*	Coefficient	*t*	*P*
Site 1						
Intercept	−2.95 (1.06)	−2.80	0.009	−0.48 (0.47)	−1.01	0.321
Invasion	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
Invasion × 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
Invasion	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
Invasion × time	−0.16 (0.04)	−4.28	<0.001	0.16 (0.04)	3.73	0.001

All sequenced DGGE bands were very closely related and within the Order Glomerales (Fig. 1; Table S3; 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; Ohsowski et al., 2014). However, we did detect biotic plant growth responses between invasion categories (see below). In addition, primer bias may have resulted in selective amplification of Glomerales (Mummey and Rillig, 2007; Krüger et al., 2012). However, field studies with *V. rossicum* targeting the internal transcribed spacer (ITS) region (Day, 2015), and the small ribosomal subunit (Bongard et al., 2013) have also shown that this plant commonly associates with Glomerales. Although these similarities with field-collected roots indicate that the identities of the AM fungi in the glasshouse were representative, it is unclear how this environmental filter could have influenced the temporal patterns in these communities.

Each *V. rossicum* plant was colonised by 7–18 AM fungal isolates (indicated by DGGE bands). This number of isolates is consistent with that found across a range of studies and ecosystems and, as such it may not be a characteristic of invasive plants alone (Öpik et al., 2009; Hausmann and Hawkes, 2010; Lekberg et al., 2012; Hart et al., 2014; Helgason et al., 2014). Invasive plants may have a competitive advantage if they are able to associate with a range of AM fungal isolates in the introduced range (Klironomos, 2002; van der Putten et al., 2007b; Pringle et al., 2009; Moora et al., 2011; Lekberg and Koide, 2014; Nuñez and Dickie, 2014). This may contribute to the absence of a strong directional temporal gradient in AM fungal community structuring in *V. rossicum* (Fig. 2), and suggests that this species will be able to invade soils that vary widely in AM fungal community composition. 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 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.

Plants growing in uninvaded soil allocated more resources towards their roots relative to their shoots (Fig. 3). These differences may have been caused by soil biotic factors because soil fertility was similar across all plots and we further controlled for soil nutrition by using one-third of field soil in the substrate. Moreover, fertiliser was applied to all pots two weeks prior to harvest 2 and the root–shoot ratios were still similar in the different invasion categories at harvest 2 (Fig. 3). However, we cannot discount the possibility that slight differences in soil nutrients may have had an effect on the biomass responses (Table S2). While there was a significant difference in AM fungal composition between treatments, other organisms were present, both antagonists and mutualists, and likely contributed to the observed growth response. For example, by targeting the AM fungal community, we may have missed critical bacterial plant-growth promoters. Future research should focus on closer linking AM fungal community structure to symbiotic functioning over time.

In conclusion, this study suggests that AM fungal communities

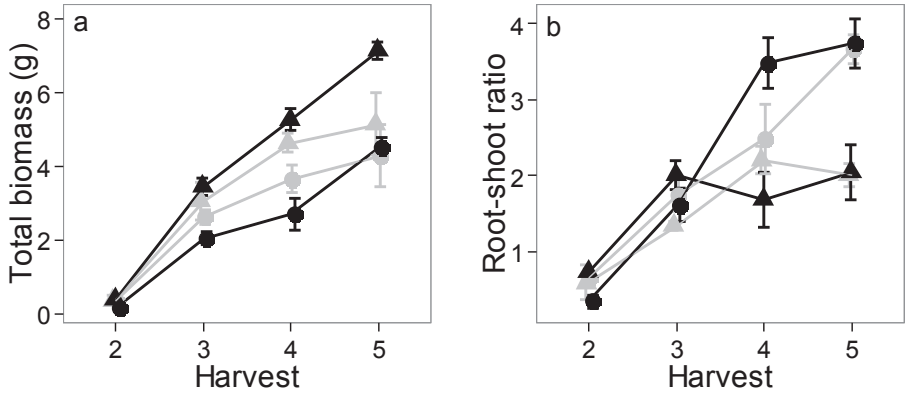

Fig. 3. (a) Total biomass (g) and (b) root–shoot ratios from harvests 2 to 5 for *Vincetoxicum rossicum* plants by site and invasion category (all root mass for harvest 1 was used for molecular work). Grey lines represent plants in Site 1, black lines represent plants in Site 2, circles represent the uninvaded treatment, triangles represent the invaded treatment. Values are means ± standard errors. Graphs were produced in R using package 'ggplot2' (Wickham, 2009).

may change in response to invasion by *V. rossicum* and that this develops over a period of time beyond the first growing season after establishment. Future work incorporating a greater number of sites across measured environmental gradients, including sites invaded by *V. rossicum* for different periods of time, is now needed to clarify the generality of the observed patterns.

Contributions

N. Day, P.M. Antunes and K.E. Dunfield designed the experiment, N. Day conducted the experiments, analysed the data and wrote the first draft of the article, and all authors discussed the results and contributed to writing the article.

All authors approved the final article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.actao.2015.06.004.

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