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Rethinking Invasion Impacts across Multiple Field Sites Using European Swallowwort (*Vincetoxicum rossicum*) as a Model Invader

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Research Article

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Abstract

European swallowwort [*Vincetoxicum rossicum* (Kleopow) Barbarich] is found in the northeastern United States and southeastern Canada. It forms dense growth patterns that reduce plant and insect biodiversity, and lab assays show that it produces allelopathic compounds that affect microbial activity. Consequently, we hypothesized that *V. rossicum* alters soil microbiome composition and activity in invaded habitats, which may impact ecosystem properties and processes. We sampled soil from a similar time point within a growing season at each of five sites in New York State where *V. rossicum* was both present and absent. We measured bacterial and fungal microbiome composition, available soil nitrogen (N), soil respiration (CO_2 flux), and soil extracellular enzyme activities. Microbial composition varied across field sites, but only fungal composition was affected by invasion. No significant differences were found between the invaded and uninvaded plots at any of the sites for available soil ammonium, nitrate, or respiration, though extractable N varied greatly between sites. Microbial hydrolytic extracellular enzyme activities suggest decreased protein degradation and increased oxidative enzyme activity with *V. rossicum* invasion, which is relevant to soil N and carbon cycling processes. Although *V. rossicum* impacted rhizosphere microbial composition and activity, it was not associated with large perturbations in ecosystem function when examined across multiple invasion sites during this short-term study.

Introduction

European swallowwort [*Vincetoxicum rossicum* (Kleopow) Barbarich] is a herbaceous plant native to Ukraine and neighboring parts of Russia, but it is invasive in southeastern Canada and the northeastern United States (Averill et al. 2010; DiTommaso et al. 2005). This perennial herbaceous vine is frequently found in abandoned fields, field edges, and woodland understories (DiTommaso et al. 2005). *Vincetoxicum rossicum* invasion manifests as dense, near-monoculture stands, resulting in decreased floral and faunal diversity (DiTommaso et al. 2005). The species is able to quickly establish due to high seed production rates, dispersal of seeds by wind, polyembryonic seeds, and tillering habit (Averill et al. 2010; Cappuccino et al. 2002; DiTommaso et al. 2005). The success of *V. rossicum* as an invader may be due to many factors of its reproductive biology and from chemical interference mechanisms, which increase its competitive ability relative to native species (Averill et al. 2010; DiTommaso et al. 2005; Douglass et al. 2010; Gibson et al. 2011; Smith et al. 2008).

There has been increasing attention to the secondary phytochemicals that plants produce for defense and offense as a means of understanding competitiveness and biological invasion. These compounds may yield a competitive edge leading to invasiveness, deemed the *novel weapons hypothesis* (Callaway et al. 2008; Cappuccino and Arnason 2006). *Vincetoxicum rossicum* has been shown to contain allelopathic chemicals, including -(–) antofine, in aboveground and belowground tissues (Douglass et al. 2010; Gibson et al. 2011). Allelopathic compounds from *V. rossicum* have been shown to suppress co-occurring plants (Douglass et al. 2010; Gibson et al. 2011) and alter rhizosphere microbiomes (Callaway et al. 2008; Mogg et al. 2008). Using disk diffusion assays of crude *V. rossicum* extract (Mogg et al. 2008) and -(–) antofine (Gibson et al. 2011), researchers found the inhibition of representative gram-positive and gram-negative bacteria and diverse fungal taxonomic groups with varying growth forms, including many *Ascomycota*. Field-based studies of *V. rossicum* allelopathic compounds

Management Implications

European swallowwort [*Vincetoxicum rossicum* (Kleopow) Barbarich] is an aggressive invader that reduces plant biodiversity and negatively impacts Monarch butterfly survival. It forms dense vegetation stands in invaded habitats, potentially impacting ecosystem properties and processes that regulate nutrient cycling, including soil microbiome structure, nutrient availability, and soil extracellular enzyme activities. Research has indicated that *V. rossicum* produces compounds that suppress the development and growth of other plants, but the effects of these compounds on soil microorganisms associated with plant roots are not well characterized. This study provides evidence that fungal soil microbiome composition was altered by *V. rossicum* invasion, but bacterial composition was unaffected, and no effects on extractable nitrogen pools or soil respiration were observed. Soil extracellular enzymes released primarily from microbial cells indicate the potential for *V. rossicum* to alter mineralization processes of carbon and nitrogen from soil organic matter. These findings are based on a preliminary assessment of microbiome structure and function, but replicated across five sites, which captures some of the potential for multisite variation in response to *V. rossicum* invasion. Restoration efforts following *V. rossicum* invasion will require more detailed and longer-term measurements of ecosystem responses across multiple sites. A more nuanced, site-specific understanding of *V. rossicum* effects on soil processes is necessary to restore ecological function to invaded landscapes.

are few, but questions remain about the efficacy of -(–) antofine in nonsterile soils and at naturally occurring concentrations as an allelopathic compound (Gibson et al. 2015). *Vincetoxicum rossicum* does not uniformly suppress the soil microbiome; conversely, positive associations with arbuscular mycorrhizal fungi (AMF; *Glomeromycota*) may increase *V. rossicum*'s competitive success as an invader (Smith et al. 2008). Thus, *V. rossicum* has complex effects on the composition of the soil microbiome when and where invasions become established.

Changes in plant community composition due to invasion can modify soil microbiome structure, resulting in alterations of microbially mediated ecosystem functions. Invasive plant species alter ecosystem functions such as nutrient cycling and soil extracellular enzyme activities (Ehrenfeld 2003, 2010; Elgersma et al. 2011). Nitrogen (N) availability is often impacted by invasive species (Castro-Díez et al. 2014; Vitousek et al. 1987). On the whole, invasive plants increase N availability and speed N cycling rates, even when invasive N-fixing plant species are excluded from meta-analyses (Castro-Díez et al. 2014; Ehrenfeld 2003). Soil bacteria mineralize organic substrates, releasing ammonium (NH_4^+) and subsequently nitrify NH_4^+ to nitrite (NO_2^-), then nitrate (NO_3^-) (Schimel and Bennett 2004); thus, changes in the bacterial microbiome can have functional consequences on inorganic N availability if there are changes in the relative abundance of bacterial ammonifiers or nitrifiers. Phosphorous (P) acquisition by plants is enhanced by associations with AM fungi in which fungal phosphatases mineralize organic P (Allison et al. 2007). Extracellular enzymes are produced by soil microorganisms to mineralize soil organic matter to access soil nutrients such as carbon (C), N, and P. Mineralized inorganic nutrients not immobilized by microorganisms are available for plant uptake, loss from the system, or further transformations. Canada goldenrod (*Solidago canadensis* L.), a North American native that is invasive in China, has been shown to affect soil microbiome composition using phospholipid fatty-acid techniques, increasing ammonification and phosphorous mineralization, while decreasing nitrification (Li et al. 2012) compared with uninvaded, native plots. Invasive Japanese barberry (*Berberis thunbergii* DC) and Japanese stiltgrass [*Microstegium vimineum* (Trin.) A. Camus] litter produced discernible changes in microbial extracellular enzyme activities compared with native species in parkland experiments, regardless of site effects (Kourtev et al. 2002).

Given that *V. rossicum* contains antimicrobial compounds in the leaves, roots, and root exudates and forms selective soil fungal associations, all of which influence rhizosphere dynamics (DiTommaso et al. 2005; Douglass et al. 2010; Gibson et al. 2011), we sought to measure soil microbial responses to *V. rossicum* invasion. We hypothesized that *V. rossicum* invasion would impact ecosystem properties and processes, with the following specificities: (1) altered soil microbiome composition, based on molecular sequencing analysis; (2) decreased soil N availability; (3) overall suppression of *Ascomycota* fungi; (4) decreased soil respiration rates; and (5) suppressed activities of different classes of extracellular enzymes in relation to decreases in soil N availability and soil respiration. The rationale for most of these predicted scenarios is based on the antimicrobial effect of the *V. rossicum* exudates on microbial composition and function. To test these hypotheses, five field sites in New York State were identified that contained similar plant communities where *V. rossicum* invasion was present or absent, and a one-time "snapshot" sampling was conducted. This approach is novel, because it combines modern molecular sequencing techniques and multisite replication of in situ conditions of sites where *V. rossicum* is both present and absent.

Table 1. Names, abbreviations, locations, and sampling dates for *Vincetoxicum rossicum* research sites around Cayuga Lake, NY.

No.	Site name	Site abbr.	Latitude	Longitude	Soil sample date	Soil respiration date
1	Edwards Lakes Cliff Nature Preserve	EL	42.522767°	-76.517759°	July 2, 2014	August 26, 2014
2	Salt Point	SP	42.540450°	-76.548438°	June 26, 2014	August 28, 2014
3	Lansing Center Trail	LCT	42.538483°	-76.497335°	June 26, 2014	August 27, 2014
4	Great Gully	GG	42.810422	-76.677183°	July 23, 2014	August 26, 2014
5	Cayuga Nature Center	CNC	42.521761°	-76.558617°	August 5, 2014	August 28, 2014

Materials and Methods

Sampling Sites

Five field sites were selected in the Finger Lakes region of New York State, near the city of Ithaca (Table 1). The sites represent early successional plant communities, with many having previous agricultural history. Sites included open fields or woodland edge containing areas both invaded (+) and uninvaded (−) by *V. rossicum*. Invaded sites contained >50% *V. rossicum* cover and uninvaded sites <1% *V. rossicum* cover by visual estimates. Field composited soil samples were kept in coolers and returned to the lab and sieved through 2-mm mesh to remove roots and organic debris within 24 h of collection. Subsamples of sieved soils were used for amplicon sequencing of bacterial and fungal composition, measurement of available soil N, and extracellular enzyme activities. The methods of the specific analyses are described in the subsequent sections. Field measurements of soil respiration were conducted once during this study. Due to limitations imposed by negotiated access to the research sites from private and public land owners, as well as equipment availability, sampling dates varied (Table 1).

Description of Sites

Edwards Lake (EL) consists of Ovid (fine-loamy, mixed, active, mesic Aeric Endoaqualfs) & Rhinebeck (fine, illitic, mesic Aeric Endoaqualfs) silt loams (2% to 6% slope), which are well drained. Salt Point (SP) has well-drained Genesee (fine-loamy, mixed, superactive, mesic Fluventic Eutrudepts) silt loams (0% to 2% slope). Lansing Center Trail (LCT) has Ilion (fine-loamy, mixed, active, mesic Mollic Endoaqualfs) silty clay loams (0% to 2% slope) that tend to be poorly drained. At LCT, adjacent sites within 14 m are active agricultural fields, though sampling sites were in restored natural areas and along old field or woodland edges. Great Gully (GG) has well-drained Honeoye (fine-loamy, mixed, semiactive, mesic Glossic Hapludalfs) silt loams (2% to 8% slope). Cayuga Nature Center (CNC) has Hudson (fine, illitic, mesic Glossaquic Hapludalfs) silty clay loams (2% to 6% slope) that are moderately well drained (USDA-NRCS 2014). Sites were selected to have similar herbaceous cover common to postagricultural early successional sites for the region, including typical species of goldenrod (*Solidago* spp.), aster (*Aster* spp.), raspberry (*Rubus* spp.), milkweed (*Asclepias* spp.), Virginia-creeper [*Parthenocissus quinquefolia* (L.) Planch.], eastern poison-ivy [*Toxicodendron radicans* (L.) Kuntze], orchardgrass (*Dactylis glomerata* L.), multiflora rose (*Rosa multiflora* Thunb.), honeysuckle (*Lonicera* spp.), sugar maple (*Acer saccharum* Marshall), white ash (*Fraxinus americana* L.), white pine (*Pinus strobus* L.), and hickory species (*Carya* spp.). *Vincetoxicum rossicum* densities at EL were among the highest observed across all sites in this study, exceeding 90% cover by visual estimates.

Microbial Community 16S rRNA Gene and Internal Transcribed Spacer Sequencing

DNA was extracted from soil samples using the MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). We amplified partial 16S rRNA genes (bacteria) and part of the fungal ITS (internal transcribed spacer) region using universal primers with the adapters required for two-step Nextera library preparation for Illumina MiSeq sequencing. For 16S rRNA gene amplification, we used the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al. 2011), and for ITS amplification, we used the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 58A2R (5′-CTGCGTTCTTCATCGAT-3′) (Gardes and Bruns 1993; Martin and Rygiewicz 2005). Amplification conditions and downstream preparation for sequencing are as described in Howard et al. (2017). For 16S rRNA gene sequencing, a 600-cycle MiSeq Reagent Kit v.3 was used, and for ITS sequencing, a 500-cycle MiSeq Reagent Kit v.2 was used (Illumina, San Diego, CA, USA). Sequencing was performed at the Cornell Genomics Facility (Ithaca, NY, USA).

For processing of the ITS sequences, we followed the modified Brazilian Microbiome Project pipeline (Pylro et al. 2014) described in Howard et al. (2017). Due to low sequencing efficiency in our 16S rRNA gene run (leading to fewer recovered sequences), we processed all 16S rRNA gene sequences in Mothur (Schloss et al. 2009) to retain at least several hundred sequences per sample. Reads were merged with *make.contigs*, primers removed with *trim.seqs* (pdiffs = 2, maxambig = 0), and sequences were aligned using *align.seqs* followed by *screen.seqs* and *filter.seqs*. The data set was reduced to only unique sequences with *unique.seqs*, and sequences with only one base pair different per 100 bp were classified as identical using *pre.cluster*. Chimeras were removed using *chimera.uchime* (dereplicate = t) followed by *remove.seqs*. Sequences were then classified with *classify.seqs* (greengenes v13_8_99), and those classified as 'Chloroplast', 'Mitochondria', 'Archaea', 'Eukaryota', or 'unknown' were removed. Operational taxonomic units (OTUs) were created at 97% sequence similarity using *cluster.split*, singletons were removed with *split.abund*, and OTUs were classified with *classify.otu*. MiSeq data have been deposited in the NCBI Sequence Read Archive and are available under the project number SRP154002.

Soil Nitrogen Availability

Soil samples were measured for extractable soil ammonium (NH_4^+) and nitrate (NO_3^-), determined as the sum of nitrite and nitrate (i.e., NO_2^- + NO_3^-), hereafter referred to as "nitrate." Extractable NH_4^+ and NO_3^- were determined using a potassium chloride (KCl) extraction method described in Drinkwater et al. (1996) with the following modifications. Following shaking with KCl, samples were filtered using an acid-washed 125-ml erlenmeyer flask, topped with a funnel and folded filter paper (Whatman No. 1, 150-mm diameter). Filtered samples were poured into 20-ml scintillation vials and stored at −20 C before analysis. Extracted samples were analyzed using a SEAL AQ2 discrete analyzer (SEAL Analytical, Mequon, WI, USA), following the EPA-150-A Rev. 2 and EPA-114-A Rev.8 protocols for measuring NH_4^+ and NO_3^-, respectively. Extractions were performed in triplicate and averaged for subsequent statistical analysis.

Soil Respiration

A CIRAS-1 portable infrared gas autoanalyzer, equipped with an SRC-1 soil respiration chamber and STP-1 soil temperature probe (PP Systems, Amesbury, MA, USA) was used to measure soil respiration. PVC rings were installed at 10 randomly selected locations at each site where *V. rossicum* was present or absent at least 72 h before sampling at each site, leaving ~10 cm of the top of the ring exposed above the soil surface [$n = 100$, 10 replicates by 2 treatments (present or absent) by 5 sites]. A flexible rubber coupling was attached to the SRC-1 and connected to each PVC

Table 2. Soil extracellular enzymes, substrates, enzyme targets, and standards measured during analysis.[a]

Enzyme assayed	Abbr.	Substrate	Enzyme targets[b]	Standard
α-Glucosidase	AG	4-MUB-α-D-glucopyranoside	Starch degradation (C)	4-MUB
β-Xylosidase	BX	4-MUB-β-D-xylopyranoside	Hemi-cellulose degradation (C)	4-MUB
β-Glucosidase	BG	4-MUB-β-D-glucopyranoside	Cellulose degradation (C)	4-MUB
β-Cellobiohydrolase	CB	4-MUB-β-D-cellobioside	Cellulose degradation (C)	4-MUB
N-acetyl glucosaminidase	NAG	4-MUB-*N*-acetyl-β-D-glucosaminide	Chitin degradation (N)	4-MUB
Leucine aminopeptidase	LAP	L-Leucine-7-amido-4-methylcoumarin hydrochloride	Protein degradation (N)	7-AMC
Acid phosphatase	AP	4-MUB-phosphate	Phosphorus mineralization (P)	4-MUB
Phenol oxidase	POX	3,4-Dihydroxy-L-phenylalanine	Lignin degradation (C,N)	DOPA
Peroxidase	PER	3,4-Dihydroxy-L-phenylalanine	Lignin degradation (C,N)	DOPA

[a]Table adapted from (German et al. 2011).
[b]Specific nutrients associated with enzyme targets are listed in parentheses: C, carbon; N, nitrogen; and P, phosphorous.

ring for sampling. The headspace volume was determined and entered into the CIRAS-1 unit before sampling.

Soil Extracellular Enzyme Activity

Soil extracellular enzymes, primarily produced by microorganisms, facilitate the decomposition of plant residues and soil organic matter (SOM), such that products become available for microbial and plant assimilation or further transformations. The potential activities of extracellular enzymes can be an indicator of the ability of a soil microbiome to transform different classes of compounds and the overall activity of microorganisms in the soil environment. Measurements of potential soil extracellular enzyme activities were performed on fresh soil within 48 h of field sampling. Refer to Table 2 for the full list of targeted extracellular enzymes and their relevance to C, N, or P cycling. The protocol was developed from German et al. (2011) and Saiya-Cork et al. (2002). Briefly, a 2- to 3-g subsample of each soil was weighed, recorded, and blended for 30 s with 150 ml 50 mM sodium bicarbonate buffer (pH 8) to achieve a suspended soil slurry. For hydrolytic enzymes, a 200-µl aliquot of slurry was added to a black 96-well, solid, flat-bottom plate (Greiner Bio-One, Monroe, NC, USA); four replicates were run for each sample per enzyme, and a separate plate was prepared for 100 mM AMC and MUB standards, respectively. A 50-µl aliquot of 200 µM fluorescently labeled enzyme substrate (Sigma-Aldrich, St Louis, MO, USA) was added to each sample. A full list of extracellular enzyme substrates is listed in Table 2. For oxidative enzymes, soil slurries were added as above to 20 wells of a white, clear-bottom 96-well plate (Greiner Bio-One, Monroe, NC, USA). Ten wells received an additional 50 µl of 50 mM buffer, while 50 µl 3,4-dihydroxy-L-phenylalanine (DOPA) was added to the remaining 10 wells. An additional 10 µl 0.3% H_2O_2 was added to half of the + buffer and + DOPA wells. Five internal standards of buffer, buffer and DOPA, and each with H_2O_2 were run on each plate. All plates were incubated at room temperature (25 C) for 3 h. With 15 min before the end of incubation, 150 µl from each oxidative well was transferred to a new plate, leaving settled sediments behind. Plates were read on a BioTek, Synergy HT Multi-Mode Microplate Reader, with Gen5 software (BioTek Industries, Winooski, VT, USA). Hydrolytic plates were read at 365-nm excitation and 450-nm emission, while oxidative plates were read at 460-nm absorbance.

Statistical Analyses

Statistical analyses were performed on the data using JMP Pro 10 (SAS, Cary, NC, USA). Data were transformed as needed to fit the assumptions of normality and homogeneity of variance; however, back-transformed means and confidence intervals are reported here. For soil extracellular enzyme activities data, outliers were removed using a Dixon's *Q*-test, then analytical replicates were averaged before further analysis. Results were analyzed using a two-level mixed model, where site is a random effect and the effect of *V. rossicum* is a fixed effect. Site is treated as random, because the sites used for this study are a sampling of sites that contain *V. rossicum*; therefore, inferences specific to each site are not of interest. Instead, the restricted maximum likelihood (REML) variance component of site indicates the percentage of unexplained variance in the model attributed to the site random effect.

$$\text{Response} = \beta_0 + \beta_1(\text{site}_{\text{random}}) + \beta_2(\text{treatment}_{\text{fixed}}) + \epsilon \quad [1]$$

The number of sequences per sample was rarefied to the lowest number available in a single sample, excluding samples in which amplification/sequencing was unsuccessful (one replicate each of 'Cayuga - *V. rossicum* absent', 'Lansing - *V. rossicum* absent', and 'Lansing - *V. rossicum* present' for 16S rRNA gene sequences, and two replicates of 'Salt Point - *V. rossicum* present' and 1 replicate of 'Gully - *V. rossicum* present' for ITS sequences). For 16S rRNA gene sequences, we rarefied to 376 sequences sample^{-1}, and for ITS sequences, we rarefied to 3,126 sequences sample^{-1}.

All downstream analyses of 16S rRNA gene and ITS sequences were performed in R. We created principal coordinate analysis (PCoA) plots of Bray-Curtis distances between sites using *vegdist* (package 'vegan') and *cmdscale* (package 'stats'). We used permutational multivariate analysis of variance (PERMANOVA) to test for significant differences in bacterial and fungal community composition by location and *V. rossicum* treatment with *adonis* (package 'vegan'). We calculated fungal and bacterial Shannon diversity for each site using *diversity* (package 'vegan'), and tested for differences based on *V. rossicum* presence or absence using

t.test (package 'stats'). We created 100% bar plots of taxonomy by site by *V. rossicum* treatment using *phyloseq* and *barplot* (package 'graphics'). We created a heat map of *Ascomycota* OTUs using average clustering of Bray-Curtis distances for both sites and OTUs, and this was visualized with *heatmap.2* (package 'gplots'). OTUs with a relative abundance less than 0.5% were not visualized, but were included in distance calculations.

Results and Discussion

Microbial Community 16S rRNA Gene and ITS Sequencing

For both 16S rRNA gene and ITS sequences, we found a significant effect of location on community composition using PERMANOVA (P <0.001 for 16S rRNA genes and ITS) (Figure 1A and B). For ITS, but not 16S rRNA genes, we found a

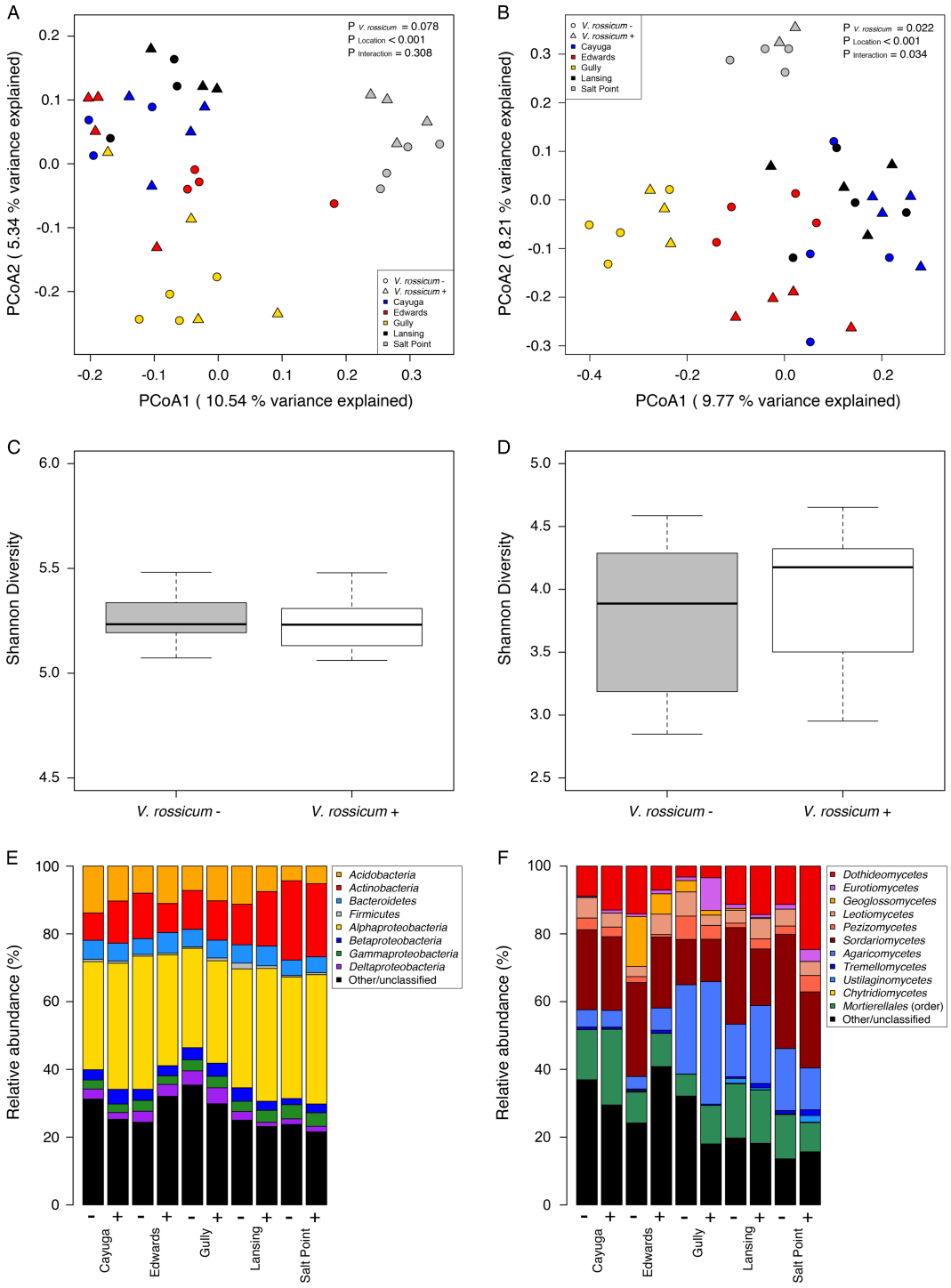

Figure 1. Soil microbial sequencing results: principal coordinate analysis (PCoA) of site location and *Vincetoxicum rossicum* treatment (+, presence; −, absence) for 16S rRNA gene (A) and fungal ITS (B); Shannon diversity for 16S rRNA gene (C) and fungal ITS (D); and percent relative abundance of major bacterial (E) and fungal (F) taxa in samples.

significant effect of both *V. rossicum* invasion (presence or absence) ($P = 0.022$) and the interaction of invasion and location ($P = 0.034$) on fungal community composition. Other studies have also shown a tighter link between plants and soil fungal communities than between plants and soil bacteria (Bell et al. 2014, 2015; Cassman et al. 2016). We found no significant differences in bacterial or fungal diversity based on the presence or absence of *V. rossicum* (Figure 1C and D). There were no consistent global trends in the promotion or inhibition of specific fungal and bacterial phyla or classes in response to *V. rossicum* presence across sites (Figure 1E and F). Results presented in Figure 1 use the same 16S rRNA or ITS sequence data set for each analysis. In the PCoA ordinations (Figure 1A and B), GG (yellow) and SP (gray) sites clustered separately from the other sites, indicating distinct microbial community compositions, especially for the fungi (Figure 1B). Fungal community composition was most impacted by *V. rossicum* presence at the EL plots (Figure 1B), which is confirmed by the shift in relative abundance of certain fungal taxa, such as an inhibition in *Dothdideomyctes* and a promotion of other/unclassified fungal organisms, compared with uninvaded sites at EL plots. However, the patterns of inhibition or promotion observed at EL plots, where the effect of *V. rossicum* was the strongest, were not consistent across the other sites (Figure 1F). Due to a high degree of redundancy within the microbiome with respect to certain functions, and site-specific differences in soil parameters that can strongly impact microbial survival (e.g. pH), different responses to treatments at different sites is not atypical (Bell et al. 2013). *Vincetoxicum rossicum* presence did not lead to consistent promotion or inhibition of specific OTUs within the *Ascomycota* (Supplementary Figure S1) in contrast to reports by Gibson et al. (2011) and Mogg et al. (2008) that *V. rossicum* crude extracts and -(–) antofine inhibited a wide array of *Ascomycota* fungal cultures in disk diffusion assays.

Soil Nitrogen Availability

Extractable soil ammonium (NH_4^+) and nitrate (NO_3^-) availability were assessed to determine whether the presence of *V. rossicum* had a significant impact on nitrogen cycling. We found no significant differences in available NH_4^+ or NO_3^- based on invasion status across sites (Table 3). The site variance component was 76.48% for NH_4^+ and 50.86% for NO_3^-, indicating high variability between sites.

Table 3. Ammonium, nitrate, and soil respiration compared between *Vincetoxicum rossicum* uninvaded (−) and invaded (+) sites.[a]

	Ammonium (mg NH_4^+-N g dry $soil^{-1}$)		Nitrate (mg NO_3^--N g dry $soil^{-1}$)		Soil respiration (g CO_2 m^{-2} h^{-1})	
	−	+	−	+	−	+
Mean	0.10	0.09	0.03	0.03	8.30	8.43
Lower 95%	0.04	0.04	0.01	0.01	4.83	4.91
Upper 95%	0.22	0.21	0.10	0.08	14.25	14.46
P	0.844		0.287		0.821	

[a]Means and upper and lower confidence intervals have been back transformed from analysis.

Table 4. Soil extracellular enzyme activity (nmol g dry $soil^{-1}$ h^{-1}) compared between *Vincetoxicum rossicum* uninvaded (−) and invaded (+) sites.[a]

Enzyme	Nutrient	*V. rossicum*	Mean activity	Lower 95%	Upper 95%	P[b]
AG	C	−	3.63	1.66	7.96	0.832
		+	3.51	1.60	7.69	
BX	C	−	4.11	1.75	9.63	0.877
		+	4.00	1.71	9.38	
BG	C	−	18.70	12.41	28.19	0.262
		+	16.55	10.98	24.94	
CB	C	−	3.68	1.74	7.76	0.385
		+	3.12	1.48	3.28	
NAG	N	−	5.23	3.58	7.63	0.171
		+	4.42	3.03	6.45	
LAP	N	−	46.12	28.47	121.22	**0.038**
		+	34.61	23.95	67.44	
AP	P	−	84.62	62.03	107.22	0.691
		+	81.60	59.00	104.19	
POX	C, N	−	161.89	97.84	225.94	**0.015**
		+	223.34	159.29	287.39	
PER	C, N	−	165.43	115.30	237.35	**0.034**
		+	196.91	137.24	282.53	

[a]Refer to Table 2 for enzyme and nutrient abbreviations. Means and lower and upper confidence intervals were back transformed after statistical analysis.
[b]Bold P-values are significant at $P = 0.05$.

Soil Extracellular Enzyme Activity

Hydrolytic extracellular enzyme activities were, in general, unaffected by *V. rossicum* invasion status, with the exception of leucine aminopeptidase (LAP) activity, which decreased significantly by 25% in the presence of *V. rossicum* (Table 4). LAP degrades proteins and is consequently implicated in soil N cycling (Sinsabaugh et al. 2008). REML variance from site ranged from 32.62% to 60.54%, indicating a wide range of between-site variability.

Phenol oxidase (POX) and peroxidase (PER) activity was significantly higher in invaded compared with uninvaded sites, by 38% and 19%, respectively (Table 4). Site variance was 26.71% for POX and 56.22% for PER. Sinsabaugh (2010) notes that it is not uncommon for oxidative exoenzyme activity to be uncorrelated with hydrolytic exoenzyme activity. Furthermore, oxidative enzyme activity can vary by 10-fold to greater than 100-fold seasonally and across submeter spatial scales (Sinsabaugh 2010). *Vincetoxicum rossicum* is known to thrive under conditions favorable to symbiotic associations with AMF (*Glomeromycota*) (Smith et al. 2008), but AMF colonization of *V. rossicum* roots was not quantified in our study. The ITS region has known limitations for identifying *Glomeromycota* as an amplicon-based DNA bar-coding technique (Stockinger et al. 2010). Oxidative enzymes have been shown to exhibit an inverse relationship with N availability (Allison et al. 2007; Sinsabaugh et al. 2005). However, our study suggests a more complicated impact on soil N

cycling, as NH_4^+ and NO_3^- availability were unaffected, but protein degradation in the form of LAP activity decreased in the presence of *V. rossicum*.

Soil Respiration

Soil respiration rates (g CO_2 m^{-2} h^{-1}) were measured to determine what effect *V. rossicum* invasion had versus existing vegetation. *Vincetoxicum rossicum* was found to have no significant effect on soil respiration rates (Table 3). The majority of unexplained variance (62.39%) was attributed to the random site variable.

In this study, we measured soil microbiome composition using bacterial and fungal molecular biomarkers, available soil NH_4^+ and NO_3^-, soil respiration, and extracellular enzyme activities to determine the impacts of *V. rossicum* invasion on ecosystem properties. Consistent with our hypothesis, we found evidence that *V. rossicum* altered soil fungal composition compared with uninvaded plots. The effect of site was also a highly significant factor for shaping both bacterial and fungal composition, highlighting the need to conduct multisite studies when assessing the effect of an invasive species such as *V. rossicum* on the structure of the soil microbiome. We hypothesized a reduction in N cycling due to reported antibacterial properties of *V. rossicum*. Though only a onetime sampling, our results show an inconsistent response, wherein a protein-degrading (LAP) extracellular enzyme activity decreased in response to *V. rossicum* invasion, but NH_4^+ and NO_3^- availability was unaffected. Interestingly, both oxidative enzymes measured (POX and PER) increased in response to *V. rossicum*. Phenol oxidases and peroxidases are frequently associated with soil fungi, typically within the divisions of *Ascomycota* and *Basidiomycota* (Allison et al. 2007; Sinsabaugh 2010). Though the ITS sequencing results did not show evidence of consistent promotion or inhibition of *Ascomycota*, *V. rossicum* was associated with significant changes to soil fungal composition compared with the uninvaded plots. We also hypothesized that soil respiration would decrease in response to *V. rossicum*, due to linkages between heterotrophic soil C and N cycles; however, we found *V. rossicum* had no effect on soil respiration.

Laboratory assays of -(−)antofine derived from *V. rossicum* tissue have shown dose-dependent effects, in which antibacterial effects require high concentrations (100 µg), while antifungal effects are seen at lower concentrations (10 µg) (Gibson et al. 2011). Measuring such effects in situ remains difficult, but Gibson et al. (2015) suggest soil microbiota may degrade or inactivate -(−) antofine, resulting in observed concentrations many times below bioactive levels. More research is needed to determine whether the observed impacts of *V. rossicum* on extracellular enzyme activities result from chemical interference with soil microbial communities, and furthermore, whether such effects are dependent on the invasion density. While only intended as a preliminary study, these results highlight the need for longer-term, multisite, in situ studies to assess the impact of *V. rossicum* invasion on ecosystem properties. Greater efforts are also needed to link shifts in microbiome structure due to invasive plant species to functional consequences in the environment.

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