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Plant invasion alters the Michaelis–Menten kinetics of microbial extracellular enzymes and soil organic matter chemistry along soil depth

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Abstract Microbial extracellular enzymes decompose distinct components of soil organic matter (SOM), thus influencing its stability. However, we lack the knowledge about how the kinetics of individual enzymes vary when multiple substrates change simultaneously. Here we used Japanese knotweed (*Polygonum cuspidatum*) invasion as a model system to explore how the Michaelis–Menten kinetics (V_{max} and k_m) of microbial extracellular enzymes vary with corresponding SOM components across soil depth (0–5, 5–10, and 10–15 cm). We hypothesized that invasion will increase the V_{max} (maximum enzyme activity) and k_m (substrate concentration at half V_{max}) of oxidative enzymes but decrease the V_{max} and k_m of hydrolytic enzymes, and that increasing soil depth will alleviate the invasion effects on the enzyme kinetics. The invasion of knotweed, which input litter rich in recalcitrant compounds, altered soil chemistry including an increase in lignin and fungal biomass compared to the adjacent non-invaded soils. The V_{max} of peroxidase, the oxidative enzyme that degrades lignin, increased in the invaded soils (0–5 cm) compared to the non-invaded soils. Among the hydrolytic enzymes, the V_{max} of *N*-acetyl-glucosaminidase which degrades chitin from fungal cell walls increased in the invaded soils (0–5 cm). However, there was no associated change in the k_m of peroxidase and *N*-acetyl-glucosaminidase under invasion, suggesting that microbes modified the enzyme production rates, not the types (isozyme) of enzymes under invasion. The V_{max} of all enzymes decreased with depth, due to the reduced substrate availability. These results highlight that the addition of relatively recalcitrant substrates due to plant invasion altered the kinetics of microbial extracellular enzymes with implications for SOM chemistry in the invaded soils.

Keywords Extracellular enzymes · Michaelis–Menten kinetics · Substrate availability · Plant invasion · Phenolics · Lignin

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Introduction

Invasion by non-native plant species poses one of the greatest threats to ecosystems around the world (Pimentel et al. 2001; Pimentel 2002; Cronk and

Fuller 2014). Apart from the well documented economic and biodiversity loss, non-native plant invasion can also influence soil organic matter (SOM) chemistry by producing physically and chemically distinct litter from the native species they displace (Elgersma et al. 2012; Tharayil et al. 2013; Tamura and Tharayil 2014; Suseela et al. 2016). As soil microorganisms deploy extracellular enzymes to degrade organic matter for resource acquisition, such changes in litter chemistry under plant invasion may alter microbial community and the amounts and types of extracellular enzymes they generate. This, in turn, may exert a positive feedback on the chemistry of SOM, creating a persistent effect of the invasive species on SOM dynamics even after their removal (Reynolds et al. 2017). However, we lack the knowledge about how the activities and kinetics of functionally different microbial extracellular enzymes would vary under non-native plant invasion. Considering that non-native plant invasion is widespread across biomes and SOM is the largest terrestrial organic carbon (C) pool, understanding microbial resource acquisition strategies under plant invasion is indispensable for projecting the stability of distinct SOM compounds in invaded ecosystems.

The activity of extracellular enzymes is often modeled by using the Michaelis–Menten kinetics (Schimel and Weintraub 2003; Wang et al. 2013; Wieder et al. 2013, 2014; Eq. 1).

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$V_{max} = V_{cat} * [E] \quad (2)$$

In Eq. 1, enzyme activity V is expressed as a fraction of the maximum enzyme activity V_{max}. As V_{max} is the product of the catalytic rate constant K_{cat} and the enzyme concentration $[E]$, more enzymes indicate greater V_{max} (Eq. 2). When substrate concentration $[S]$ is relatively small, V is lower than V_{max} and increases with $[S]$. At relatively high $[S]$, all active sites of enzymes are occupied with substrates, thus V approaches V_{max}. By definition, k_m is the substrate concentration at which half of the V_{max} is reached. The k_m value represents the inverse affinity of the enzyme to substrate. That is, the lower the k_m, the better the enzyme binds to its substrate. Under laboratory conditions where a single enzyme at fixed concentration reacts with its corresponding substrate without microorganisms, the Michaelis–Menten parameters (V_{max} and k_m) describe the intrinsic enzyme properties, unique to each enzyme–substrate pair.

However, when microbes are included in the system, the Michaelis–Menten parameters reflect microbial modifications in the amounts and types of an enzyme (i.e. isozyme). When microbes invest their energy and extant resources in enzyme production, there is a trade-off between the amount (V_{max}) and efficiency (k_m) of the enzyme (Benner 1989; Allison et al. 2011, 2014; German et al. 2011). Generating more efficient enzymes is expensive because microbes need extra machinery to alter enzyme's hydrophobicity, the number and types of chemical bonds (hydrogen bond and electrostatic bond) to achieve a stable substrate-enzyme complex. Thus, microbes are likely to adjust their enzyme productions (amounts and types) when substrate quantity and quality vary. For example, when substrates are abundant, it may be advantageous for microbes to produce less efficient (high k_m) but more extracellular enzymes (high V_{max}), because the rate-limiting factor is the amount of enzymes, not the substrate. Consistent with this prediction, Loeppmann et al (2016) recently demonstrated that microbes inhabiting soils rich in labile C exhibit higher V_{max} and k_m of hydrolytic enzymes than those in soils with low labile C. Alternatively, when changes in substrate quantity and composition modify microbial community composition, it could lead to the production of different isoforms of enzymes with distinct k_m (Stone et al. 2012).

Invasive species can indirectly influence enzyme kinetics if changes in substrate quality and quantity alter microbial community composition and the associated enzyme production. For example, many invasive species generate litter rich in phenolic compounds such as lignin. Because the production of oxidative enzymes that degrade lignin is largely limited to fungi (Sinsabaugh 2010), increasing lignin can stimulate fungal growth (Six et al. 2006; Tamura and Tharayil 2014; Suseela et al. 2016). Microbial community with relatively high fungal abundance may generate more oxidative enzyme (higher V_{max}) to decay lignin, while decreasing efficiency (higher k_m) due to the trade-off.

Invasive species can also directly alter extracellular enzyme kinetics via inhibition of enzymes by phenolics. Non-native plant species often generate litter rich in extractable phenolics including tannins (Liao et al. 2006; Li et al. 2010; Yuan et al. 2014; Suseela et al.

2016). The phenolics can serve as an inhibitor of enzyme activity. Tannins can directly bind to enzymes and suppress the catalytic activity via changes in enzyme solubility, secondary and tertiary structure as well as hydrophobicity (Rohn et al. 2002; Joanisse et al. 2007; Ximenes et al. 2011). This direct enzyme-phenolics interaction can decrease V_{max} of all types of enzymes, with k_m reduced (uncompetitive inhibition) or unchanged (non-competitive inhibition). In uncompetitive inhibition, phenolics can only bind to the enzyme–substrate complex. As a consequence of Le Chatelier's principle, the equilibrium of $E + S \leftrightarrow ES$ shifts to the right, reducing k_m. Also, the binding of phenolics to the enzyme–substrate complex would prolong the time for the product to leave the active site of the enzyme (decreasing V_{max}). In non-competitive inhibition, phenolics can bind to either enzyme alone or enzyme–substrate complex. Thus, the equilibrium $E + S \leftrightarrow ES$ would stay the same (no change of k_m), but it still takes longer for the product to leave the ES complex (decreasing V_{max}). Given the differential roles that altered litter quantity and quality play in modulating enzyme kinetics, it is important to explore individual SOM components and corresponding enzyme activity along with changes in the microbial community under invasion.

The distribution of distinct SOM can vary differentially with soil depth with the potential to alter the Michaelis–Menten kinetics of enzymes across depths. Generally, the abundance of SOM is highest at surface soil due to higher plant leaf litter input, and decreases with depth as the distance from the input source increases (Angst et al. 2016; Billings et al. 2018). Therefore, the effects of plant invasion on enzyme kinetics may be confined to surface soils, not extended into soils at increasing depths. Alternatively, SOM at increasing depth may be associated with more mineral and hence have higher stability, which can alter enzyme kinetics (Loeppmann et al. 2016).

To evaluate the enzyme kinetics under plant invasion, we chose Japanese knotweed (*Polygonum cuspidatum*) invasion as a model system, because *P. cuspidatum* invasion creates a novel substrate field by generating physically and chemically distinct litter inputs than the native species they displace (Dassonville et al. 2007; Fan et al. 2009; Suseela et al. 2016). Our study site in Massachusetts has been invaded by *P. cuspidatum* for > 20 years. Previous studies conducted at this site revealed that *P. cuspidatum* input litter rich in phenolic compounds including lignin and tannins (Suseela et al. 2016), resulting in increased SOM content and altered SOM composition (Tamura and Tharayil 2014; Tamura et al. 2017). The invasion of *P. cuspidatum* also increased fungal biomass and decreased bacterial biomass at this study site (Tamura and Tharayil 2014; Suseela et al. 2016), in Massachusetts and several other *P. cuspidatum* invaded sites across the eastern US, where the invaded soils had a uniform fungal community composition than the adjacent non-invaded soils. Decomposing *P. cuspidatum* leaves had more fungal colony-forming units than the litter of native herb plants (Mincheva et al. 2014). The shift in the microbial community towards a more fungal dominated system may also alter the kinetics of enzyme activity in the invaded system with implications for SOM decomposition.

Based on the changes in the substrate availability under *P. cuspidatum* invasion and the roles of phenolics in modulating enzyme activity, we hypothesized that the increase in lignin under invasion would enable oxidative enzymes to overcome the enzyme-phenolics inhibition and the decrease in labile C and phenolics inhibition would negatively influence hydrolytic enzyme kinetics. Specifically, we hypothesized that plant invasion will increase the V_{max} and k_m of oxidative enzymes but decrease the V_{max} and k_m of hydrolytic enzymes. We also hypothesized that the invasion effect on V_{max} and k_m would decrease with increasing soil depth. Here, we determined the Michaelis–Menten kinetics of hydrolytic (acid phosphatase [AP], β-glucosidase [BG], *N*-acetyl-glucosaminidase [NAG]) and oxidative (peroxidase [PER]) extracellular enzymes and the corresponding substrate quantities (acid phosphatase/organic phosphorus, β-glucosidase/cellulose, *N*-acetyl-glucosaminidase/chitin, and peroxidase/lignin) when substrate field varies with plant invasion and soil depth.

Methods

Soil sampling and initial physicochemical characteristics

We chose our study site in Amherst, MA (42° 24′ N, 72° 31′ W), where *P. cuspidatum* has invaded native

ecosystems for more than 20 years (Tharayil et al. 2013; Tamura and Tharayil 2014) with little apparent disturbance. The invaded and the adjacent, non-invaded stands experience the same climate and exhibit similar soil properties such as pH, bulk density, texture, and soil moisture (Tamura and Tharayil 2014; Suseela et al. 2016; also unpublished data in this study). However, the long history of invasion led to distinct biological and chemical properties between the invaded and the non-invaded stands (Tharayil et al. 2013; Tamura and Tharayil 2014; Suseela et al. 2016). The dominant vegetation in the non-invaded stand was *Dactylis glomerata, Setaria viridis, Solidago sp., Galium aparine, Rumex crispus, Ambrosia sp., Cyperus rotundus, Epilobium, Fraxinus pennsylvanica, Daucus carota, Ipomoea sp., Arisaema triphyllum, Viola sp., Hypericum perforatum, Impatiens capensis, Oenothera biennis, Erigeron annuus, Plantago lanceolata, Plantago major, Oxalis stricta*, and *Trifolium pratense*. Similar to most studies in invasion ecology where invasion is not experimentally manipulated, we cannot completely rule out the possibility that the difference between invaded and non-invaded stands existed before invasion rather than a consequence of the invasion. However, the uniform chemistry and soil microbial community that occurred in *P. cuspidatum* invaded soils in geographically distinct locations across the eastern US including the same study site reduced the concern that other causes could have resulted in the difference between the invaded and non-invaded stands (Suseela et al. 2016). Therefore, even though we acknowledge that our sampling was limited to one study site, we believe that the results obtained here are largely due to the invasion of *P. cuspidatum*, not by chance, and are generally applicable to other *P. cuspidatum* invaded soils.

Using transect sampling, we collected six soil cores (5 cm diameter) from 0 to 5, 5 to 10, and 10 to 15 cm in invaded and adjacent non-invaded stands, respectively in August 2018 (n = 6). First, we set up a 15 m primary transect along the invading front, then established six, 16 m secondary transects, each separated by 3 m, centered on and perpendicular to the primary transect. At each secondary transect, soils at 8 m distance (toward invaded and non-invaded zones, respectively) from the primary transect were collected. All soils were shipped immediately to Clemson University in a cooler and stored at − 20 °C until analysis. Roots, litter, and stones were manually removed from the soil samples before further analysis.

Soil chemistry

Soil organic matter (SOM)

The SOM content was quantified using loss on ignition method. Briefly, we dried 1 g of fresh soil in a crucible at 105 °C and 600 °C overnight sequentially and the difference in the sample weight between 105 °C and 600 °C was used for calculating the content of SOM.

Dissolved organic carbon (DOC)

We extracted the DOC by mixing 5 g of soil with 40 mL of 2 M KCl. The soil slurry was gently shaken on a rotary shaker for 1 h and centrifuged at 2,500 rpm for 10 min. The supernatant was filtered through a 0.45 µm syringe filter (Aqua 30, Whatman). The filtrate was frozen at − 20 °C and analyzed on a Shimadzu TOC-L analyzer.

Soil phenolics

We extracted soil phenolics using base hydrolysis as described in Wang et al. (2015). We added 6 mL of 1 M NaOH to 1 g of soil, vortexed, and incubated at 90 °C for 1 h. After cooling in ice for 10 min, the tubes were centrifuged at 2500 rpm for 5 min and 4 mL of supernatant was transferred to new tubes. We added 5 µL of internal standard (*trans*-cinnamic acid and ethyl vanillin, each at 400 mg/L) and 2 mL of 50% HCl to obtain a pH < 2. After cooling in ice for 5 min, the tubes were centrifuged at 2500 rpm for 5 min and 4 mL of the supernatant was transferred to new tubes. We added 2 mL of cold ethyl acetate to the supernatants and cooled the samples in ice for 5 min. Liquid–liquid extraction of phenolics was facilitated by mixing the tubes on a rotary shaker for 15 min and cooling them in ice for 10 min. After centrifugation at 2500 rpm for 5 min, 1 mL of the upper ethyl acetate layer containing phenolics was transferred to 2 mL GC vials and stored at − 20 °C until analysis. We transferred 200 µL of the sample to 300 µL insert/GC vials and dried under Ar. The samples were silylated via reaction with 100 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane at 65 °C for 25 min. The derivatized samples were

analyzed using a gas chromatography-mass spectrometry (GC–MS; Agilent 7980A GC system coupled with a 5975 C Series quadrupole mass analyzer, Agilent) with a split ratio of 20.

Lignin, chitin, and cellulose

We employed pyrolysis-GC–MS for assessing the relative abundance of cellulose, chitin, and lignin. Pyrolysis-GC–MS can help characterize SOM chemistry without any chemical extraction. Pyrolysis uses thermal energy to break down SOM compounds to smaller molecules, which in turn are separated and detected on a GC–MS. Because each fragment exhibits a fingerprint of the parent SOM compound, pyrolysis-GC–MS enables molecular identification of individual SOM compounds in heterogeneous soil (Saiz-Jimenez and de Leeuw 1984; Hempfling and Schulten 1990; Nierop et al. 2005; Buurman et al. 2007; Pold et al. 2017). In this study, the isothermal furnace method was used to perform pyrolysis on soil samples (CDS 5150, CDS inc.). A total of 20–30 mg of soil samples were weighed into quartz tubes along with 4 µL of the internal standard (1 mM fluoranthene in 50:50 (v/v) ethanol: chloroform). Samples were heated at 500 °C for 60 s and the pyrolysates were carried to a GC–MS (Agilent 7980A/5975C Series, Agilent) in He. Samples were analyzed with a split ratio of 25, mass range m/z of 50–500, and ionization energy 70 eV. Peaks were deconvoluted and extracted using AMDIS v3.2 and compared to the reference spectra in the National Institute of Standards and Technology mass spectral libraries and literature (van der Kaaden et al. 1984; Nierop et al. 2005; Buurman et al. 2007; Furuhashi et al. 2009; Mattonai et al. 2016; Zhang et al. 2017). After correcting sample matrix effects on peak area using the internal standard, we grouped each pyrolysate into three categories (i.e., lignin, chitin, and cellulose, Table S1) and summed the relative peak area. Our results do not provide the absolute quantification of each compound, but rather allows us to compare the relative abundance of each compound across samples.

Organic phosphorus

We determined organic phosphorus content by subtracting inorganic phosphorus content from total phosphorus content (De Schrijver et al. 2012). For each sample, we subsampled two 1 g of soil, one for total phosphorus and the other for inorganic phosphorus. Soils for total phosphorus were ignited at 600 °C for 1 h to release phosphorus from an organic entity (Legg and Black 1955; Saunders and Williams 1955). We added 30 mL of 1 M HCl to the inorganic phosphorus and the total phosphorus subsamples, and these subsamples were shaken at 200 rpm for 16 h for inorganic phosphorus extraction. The extractants were centrifuged at 3000 rpm for 20 min and the supernatants were passed through a 0.45 µm syringe filter (Aqua 30, Whatman). The filtrates were stored at − 20 °C until analysis. After thawing, samples were analyzed for orthophosphate on a micro-segmented flow analyzer (A2, Astoria-Pacific) using a colorimetric method (absorbance change at 660 nm). The difference in orthophosphate between the total phosphorus and total inorganic phosphorus in samples was considered as total organic phosphorus (De Schrijver et al. 2012).

Fungal biomass-ergosterol

We used base hydrolysis to extract ergosterol for the quantification of live fungal biomass (Tamura and Tharayil 2014; Ekblad and Mikusinska 2016). A total of 6 g of fresh soil was mixed with 13 mL of 0.14 M KOH in methanol in glass tubes. The tubes were covered with aluminum foil and incubated in a heat block at 85 °C for 50 min. We transferred 9 mL of the supernatant to new tubes and added 4 mL of 1 M HCl. After centrifugation at 2500 rpm for 10 min to precipitate interference, 12 mL of the supernatant was transferred to new tubes. We added 2 mL of hexane to samples for liquid–liquid extraction. Samples were dried under Ar and silylated with 100 µL *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane at 60 °C for 40 min. The derivatized samples were analyzed using GC–MS (Agilent 7890A/5975C). The ergosterol standards were prepared in chloroform at 0, 6, 13, 25, 50, and 100 ppm concentrations.

Extracellular enzyme assays

We quantified the enzyme activity (V) and Michaelis–Menten kinetics of four microbial extracellular enzymes relevant to soil C dynamics such as using substrate analogs: β-glucosidase (cellulose decay)

with 4-methylumbelliferyl beta-D-glucopyranoside (MUB-BG; M3633, Sigma-Aldrich), *N*-acetyl-glucosaminidase (chitin decay) with 4-methylumbelliferyl *N*-acetyl-beta-D-glucosaminide (MUB-NAG; M2133, Sigma-Aldrich), acid phosphatase (organic phosphorus decay) with 4-methylumbelliferyl phosphate (MUB-AP; M8883, Sigma-Aldrich), and peroxidase (lignin decay) with 3,3′,5,5′-tetramethylbenzidine (TMB; cat.229280050, Acros). All enzyme assays were performed using a microplate reader (Synergy Mx, Biotek).

We prepared soil slurries by mixing 1 g soil with 125 mL of 50 mM sodium acetate buffer at each soil pH and blending for 30 s. For hydrolytic enzyme assays, we used black 96 well plates for fluorescence detection at 360 nm extinction and 450 nm emission wavelengths. MUB-substrates were prepared at 0, 25, 50, 100, 200, 400, and 600 μM in deionized water for each enzyme–substrate pair to obtain the Michaelis–Menten plot. For each soil sample, we used one microplate, including standards (10 μM of 4-methylumbelliferone (MUB; M1381, Sigma-Aldrich), quench control, soil control, substrate control, and assay wells (Table S2). We quantified the release of fluorescent MUB tag from the substrate analog, five times for 75 min. The fluorescence emission was corrected using quench control, substrate control, and soil control, and transformed to unit mol of MUB using standard (Min et al. 2014, 2019). After checking the linearity of the amount of MUB released (fluorescence level) over time (Supplementary Information (SI) 1), the slope was used to calculate enzyme activity (μmol/h/g dry soil) at each MUB-substrate concentration.

For oxidative enzyme assays, clear 96 well plates were used for quantifying absorbance at 450 nm. We chose TMB as a substrate for peroxidase, as it is very sensitive to oxidative enzymatic activity, exhibits linear changes in absorbance with time, and exhibits the Michaelis–Menten kinetics (Johnsen and Jacobsen 2008; Singh et al. 2013; Triebwasser-freese et al. 2015), making it suitable for this study. We prepared TMB at 0, 160, 320, 800, 1600, 2400, and 2800 μM in 50 mM citric acid to prevent auto-oxidation (Perron and Brumaghim 2009). Each clear plate includes phenol oxidase/mineral control, soil control, substrate control, and assay wells (Table S3). A standard plate was prepared separately from assay plates. The absorbance was measured four times for 25 min and the reactions were stopped at each time point by adding 70 μL of 0.2 M H_2SO_4. The net peroxidase absorbance was converted to unit mol of product generated (net peroxidase absorbance = 0.0257 × TMB content (μmol) + 0.1963). The slope between the absorbance and elapsed time was used to calculate enzyme activity (μmol/h/g dry soil) at each TMB concentration (Supplementary Information (SI) 1).

Statistical analyses

All data were checked for normality and log-transformed if necessary, to meet the assumption of normal distribution. We estimated V_{max} and k_m by fitting the Michaelis–Menten model to enzyme activity V in R. The effects of invasion, depth, and the interaction of invasion and depth on the Michaelis–Menten parameters, soil chemistry, and ergosterol were tested using a mixed-effects restricted maximum likelihood (REML) model. Fixed factors were invasion, depth, and the interaction between invasion and depth, and random factor was soil core. When there were significant effects of invasion and depth at $\alpha = 0.05$, Tukey's HSD test was employed to discern the differences among individual groups. Non-metric multidimensional scaling was used to visualize and separate the effects of invasion and depth on overall soil chemistry. Figures were prepared using Sigmaplot 12.0 (Systat Software) and statistical analyses were performed using R (R core team, version 3.6.3).

Results

Soil chemistry and fungal biomass

Non-metric multidimensional scaling analysis of soil chemistry showed that the invaded soils clustered separately from the non-invaded soils (Fig. 1). While soil chemistry in the non-invaded soils exhibited a relatively uniform distribution across depth, soil chemistry in the invaded soils at 0–5 cm and 5–10 cm depth were clearly separated from that at 10–15 cm.

Total SOM content was influenced by the interaction between invasion and depth ($p = 0.024$, Fig. 2a, Table S4). The *P. cuspidatum* invaded soils tended to have higher SOM compared to the adjacent non-invaded soils at 0–5 cm ($p = 0.08$). However, at

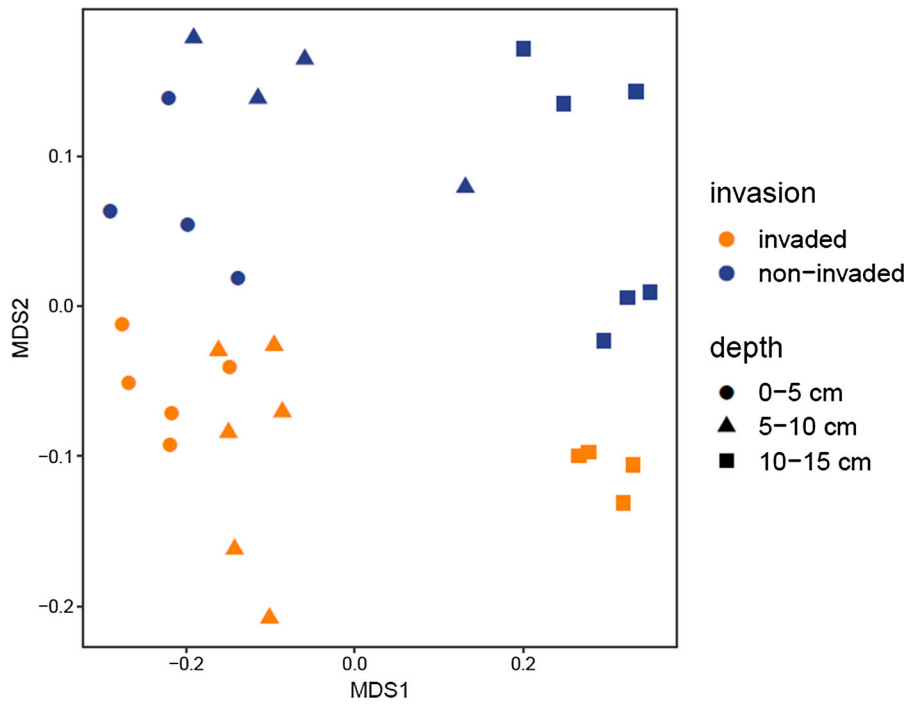


Fig. 1 Non-metric multidimensional scaling of soil chemistry (n = 6). Different colors represent the invaded (orange) and the non-invaded soils (blue) and different symbols indicate different soil depth (circles for 0–5 cm, triangles for 5–10 cm, and squares for 10–15 cm). (Color figure online)

5–10 cm and 10–15 cm, the SOM content was similar between the invaded and non-invaded soils. Overall, depth decreased SOM content both in the invaded and non-invaded soils ($p < 0.001$, Fig. 2a). There was a positive relationship between DOC and SOM (Pearson's correlation = 0.46, $p = 0.0058$, Table 1). Even when DOC content was corrected by SOM content, invasion still significantly elevated DOC content at all depths ($p = 0.02$, Fig. 2b; also see Supplementary Fig. 1a). Soil phenolics were not influenced by invasion, but significantly changed with depth ($p = 0.01$, Fig. 2c).

Invasion and depth interactively influenced the relative abundance of lignin ($p = 0.02$, Fig. 3a). The invaded soils exhibited a greater lignin content at 0–5 cm ($p = 0.06$) and 5–10 cm ($p = 0.03$) than the non-invaded soils. The relative abundance of chitin varied with depth, with soils at 0–10 cm exhibiting higher chitin content than soils at 10–15 cm ($p = 0.005$, Fig. 3b). Chitin content tended to be higher in the invaded soils than the non-invaded soils at 0–5 cm and 5–10 cm, but the differences were not significant. Neither invasion nor depth significantly influenced the relative abundance of cellulose (Fig. 3c). The organic phosphorus concentration was influenced by depth, with increasing values along soil depth ($p = 0.002$, Fig. 3d). The effects of invasion, depth, and the interaction of invasion and depth on the relative abundance of enzyme substrates per g soil (lignin, chitin, cellulose, organic phosphorus) exhibited similar patterns as observed for those per g SOM (Supplementary Fig. 2).

Ergosterol, the live fungal biomass index, was interactively influenced by invasion and depth ($p = 0.002$, Fig. 4). At 0–5 cm, ergosterol was 37% higher in the invaded soils than the non-invaded soils. In contrast, at 5–10 and 10–15 cm, the ergosterol content did not differ between the invaded and non-invaded soils. Ergosterol exhibited a strong positive relationship with lignin content (Pearson's correlation = 0.79, $p < 0.001$, Table 1), but had no relationship with chitin content (live and dead fungal cell wall; Pearson's correlation = 0.16, $p = 0.34$, Table 1).

Enzyme activity *V* and Michaelis–Menten parameters

The activity of PER (peroxidase, oxidative enzyme) was higher under invasion at all depths (Fig. 5a–c). However, hydrolytic enzymes showed different patterns from PER. For example, invasion and depth interactively influenced the activity of NAG and BG

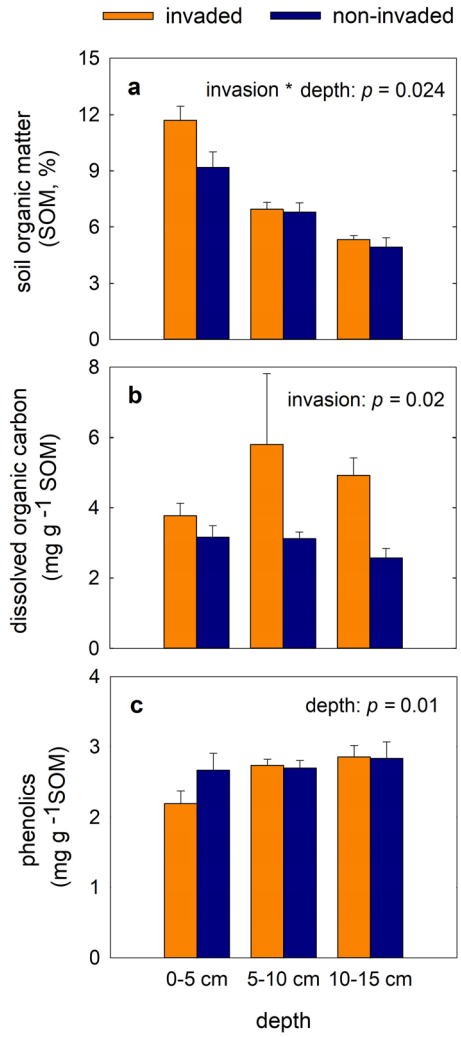


Fig. 2 Soil organic matter (**a**), dissolved organic carbon (**b**) and phenolics (**c**) under invaded (orange bars) and the non-invaded soils (blue bars) at 0–5, 5–10, and 10–15 cm (n = 6). The concentration of dissolved organic carbon and phenolics was corrected by soil organic matter content (see Supplementary Fig. 1 for non-corrected values). Significant difference among individual groups was tested at $\alpha = 0.05$. (Color figure online)

(Fig. 5d–i). NAG and BG activity in the invaded soils was higher than that in the non-invaded soils at 0–5 cm (Fig. 5d, g), lower at 5–10 cm (Fig. 5e, h), and similar at 10–15 cm (Fig. 5f, i). Unlike NAG and BG, the activity of AP did not differ between the invaded and non-invaded soils, but decreased with depth (Fig. 5i, j).

Both invasion and depth significantly influenced the V_{max} of PER without an interactive effect (Fig. 6a). The V_{max} of PER increased under invasion and decreased with depth. Across invasion and depth, changes in the V_{max} of PER had a strong, positive relationship with the changes in the relative lignin content (Pearson's correlation = 0.45, $p = 0.0061$, Table 2).

Invasion and depth interactively influenced the V_{max} of NAG and BG (Fig. 6b, c). At 0–5 cm, the V_{max} of NAG was 4.6 times higher in the invaded soils than the non-invaded soils (Fig. 6b). In contrast, the V_{max} of BG was greater in the non-invaded soils at 5–10 cm and 10–15 cm than that in the invaded soils (Fig. 6c). The V_{max} of AP decreased with increasing depth ($p < 0.001$) but did not vary with invasion (Fig. 6d).

The k_m of PER did not vary with invasion or depth (Fig. 6e). But when the data was pooled across invasion and depth, the k_m of PER had a significant, positive relationship with the relative abundance of lignin (Pearson's correlation = 0.39, $p = 0.0174$, Table 2). Invasion did not alter the k_m of either NAG or BG (Fig. 6f, g), but depth decreased the k_m of NAG ($p = 0.004$, Fig. 6f). The k_m of AP varied with invasion, where the invaded soils had higher k_m compared to the adjacent non-invaded soils ($p = 0.04$, Fig. 6g).

Discussion

By quantifying the Michaelis–Menten kinetics of distinct enzymes and corresponding substrate concentrations in soil, we demonstrate that plant invasion increased the productions of the oxidative enzyme (PER) and nitrogen-acquiring enzyme (NAG) while maintaining the enzyme efficiency similar at surface soils, and that the effects of invasion on the Michaelis–Menten parameters varied with soil depth. Our study is unique since we examined the actual enzyme substrates (i.e., lignin, chitin, cellulose, organic phosphorus), not bulk SOM content, dissolved nutrients (e.g., dissolved organic carbon, NO_3^-, NH_4^+), or operationally defined SOM chemistry, and we assessed enzyme activity along a substrate gradient, not at one substrate concentration. Thus, our approach allowed us to quantitatively link enzyme kinetics to the corresponding substrates and identify what mechanisms drive changes in microbial enzyme kinetics.

Table 1 Pearson's correlation coefficient (r) among soil chemistry index and ergosterol

	SOM	DOC	Phenolics	Lignin	Chitin	Cellulose	Organic P	Ergosterol
SOM		**0.46**	**0.87**	**0.83**	**0.43**	**0.77**	0.11	0.67
DOC			**0.60**	**0.44**	0.31	**0.34**	0.05	**0.45**
Phenolics				**0.68**	**0.37**	**0.65**	0.23	**0.65**
Lignin					0.25	**0.53**	0.25	**0.79**
Chitin						0.29	− 0.11	0.16
Cellulose							0.09	**0.39**
Organic P								0.34
Ergosterol								

Bold: $p < 0.05$

SOM soil organic matter, *DOC* dissolved organic carbon, *P* phosphorus

Invasion indirectly modifies enzyme activity and Michaelis–Menten kinetics

While changes in the Michaelis–Menten parameters can occur via either direct phenolics-enzyme interaction or indirect substrate effects on microbial enzyme production, our data suggest that the indirect effects were dominant in this study. First, we observed different enzyme activity V of PER, NAG, and BG between the invaded and the non-invaded soils at 0–5 cm (Fig. 5a, d, g) in spite of the similar phenolics (Fig. 2c, Supplementary Fig. 1b). If phenolics directly inhibited enzyme activity via phenolics-protein interaction, we should have observed a similar degree of inhibition for PER, NAG, and BG in the invaded and non-invaded soils, which was not the case. However, different classes of phenolics (e.g. flavonoids, monophenolics) may differentially affect the potential activity of peroxidase (Suseela et al. 2016). It was not surprising to observe similar levels of phenolics between the invaded and the non-invaded soils in this study, given that we collected soils in August (end of the growing season). Previous studies from the same site showed that the pool size of phenolics was higher under invasion in spring (Tharayil et al. 2013; Suseela et al. 2016), but that phenolics content did not differ between the invaded and non-invaded soils in July (Tharayil et al. 2013). Thus, it should be noted that the enzyme activites and subsequent soil C and N cycling may exhibit seasonal variation in the invaded sites (Tharayil et al. 2013). Second, the changes in the Michaelis–Menten parameters for PER (Fig. 6a, e) and NAG (Fig. 6b, f) at 0–5 cm (same k_m and higher V_{max}) did not follow those under typical enzyme inhibition scenarios. For example, when phenolics bind to the enzyme, it can modify enzyme solubility, secondary and tertiary structure, and hydrophobicity (Rohn et al. 2002; Joanisse et al. 2007; Ximenes et al. 2011). These changes in the structural and chemical properties of the enzyme can lower enzyme activity noncompetitively (same k_m and lower V_{max}) or uncompetitively (lower k_m and lower V_{max}; Waldrop 2009). However, we did not observe such changes in the Michaelis–Menten parameters of PER and NAG, suggesting that the changes in the activity of PER and NAG under invasion may not be due to the direct inhibition of enzymes by phenolics. In contrast, the same k_m and lower V_{max} of BG in the invaded soils imply that phenolics may have bound to the enzyme and enzyme–substrate complex, inhibiting noncompetitively (Fig. 6c, g).

Instead, invasion was more likely to influence enzyme kinetics via changes in the microbial community and associated enzyme production in this study. The strong, positive relationships between ergosterol and lignin (Table 1) and between ergosterol and V_{max} of PER ($r = 0.73$, $p < 0.001$), and the increase in ergosterol under invasion at 0–5 cm (Fig. 4a) suggests that the increased lignin input under *P. cuspidatum* stimulated fungal growth, which in turn, produced PER for decaying lignin. In addition, a significant, positive relationship between ergosterol and V_{max} of NAG ($r = 0.87$, $p < 0.001$) also indicates that fungal growth enhanced the production of NAG. Plant invasion often modifies soil microbial community composition (Batten et al. 2006; Liao and Boutton 2008; Elgersma and Ehrenfeld 2011). It has been reported from the same study site that the invasion of *P.cuspidatum* increased fungal biomass but decreased bacterial biomass (Tamura and Tharayil 2014; Suseela et al. 2016), and fungal community composition was significantly different between the invaded and the non-invaded soils (Suseela et al. 2016). The *P. cuspidatum* invaded soils across several sites in the

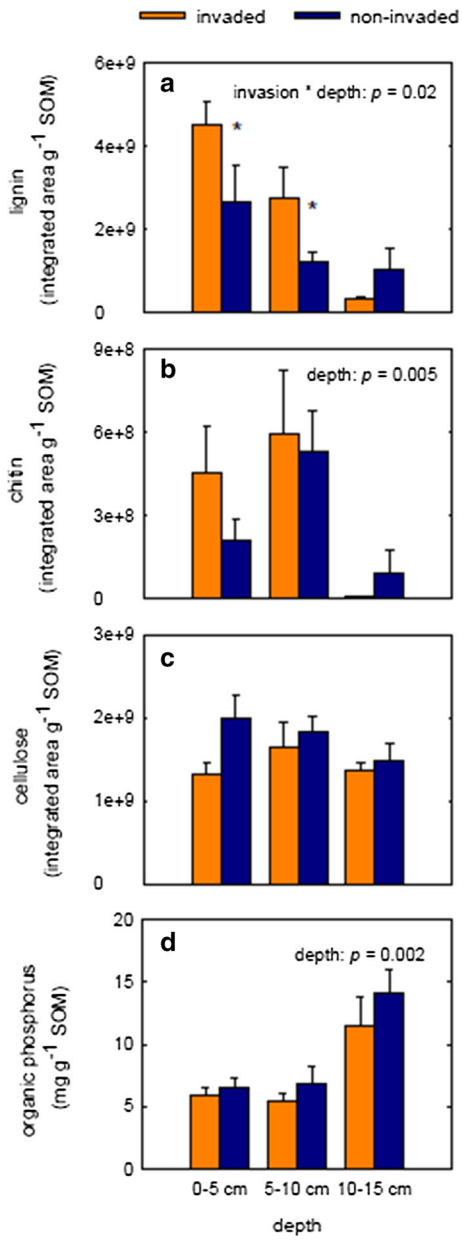


Fig. 3 Relative abundance of lignin (**a**), chitin (**b**), cellulose (**c**) and organic phosphorus concentrations (**d**) under invaded (orange bars) and the non-invaded soils (blue bars) at 0–5, 5–10, and 10–15 cm (n = 6). Significant difference between the invaded and the non-invaded soils was marked with an asterisk at $\alpha = 0.05$. Integrated area refers to the area under the corresponding peaks from the chromatogram. Enzyme substrate concentration was normalized by soil organic matter content (see Supplementary Fig. 2 for non-corrected values). (Color figure online)

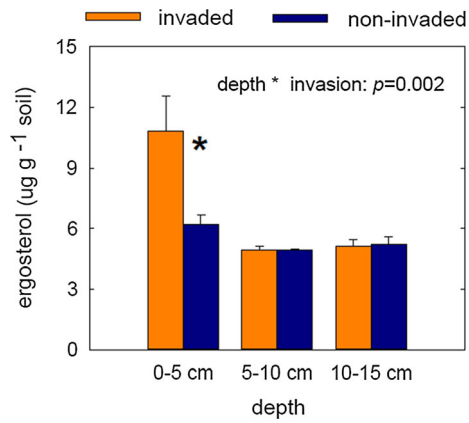


Fig. 4 Ergosterol concentration as an estimate of live fungal biomass under invaded (orange bars) and the non-invaded soils (blue bars) at 0–5, 5–10, and 10–15 cm (n = 6). Significant difference between the invaded and the non-invaded soils was marked with asterisk at $\alpha = 0.05$. (Color figure online)

eastern US had a uniform fungal community compared to the adjacent non-invaded stands. The increase in fungal biomass and uniform fungal community in *P. cuspidatum* invaded stands is potentially due to the input of chemically distinct litter rich in recalcitrant compounds (Suseela et al. 2016; Tamura et al. 2017). Due to the differences in the biomass stoichiometry, nutrient use efficiency, and metabolic pathways, different microbes generate a unique combination of extracellular enzymes to meet their distinct resource demand (Anderson and Domsch 2010; Keiblinger et al. 2010; Crowther and Bradford 2013). For example, increased recalcitrant C requires the activity of oxidative enzymes that are generated by fungi (Sinsabaugh 2010). This may be due to relatively high biomass C:N ratio (thus lower nitrogen demand) and greater C use efficiencies of fungi (Six et al. 2006; Keiblinger et al. 2010), conferring fungi to grow relatively better on recalcitrant C than bacteria. Thus, plant invasion seemed to alter the Michaelis–Menten parameters via favoring fungi over bacteria and influencing microbial communities' enzyme production in this study.

Microbes under invasion alter the production rate of enzyme, not the type of enzyme

Modification of V_{max} with little to no changes in k_m under plant invasion highlights that microbial communities that have diverged over 20 years of invasion

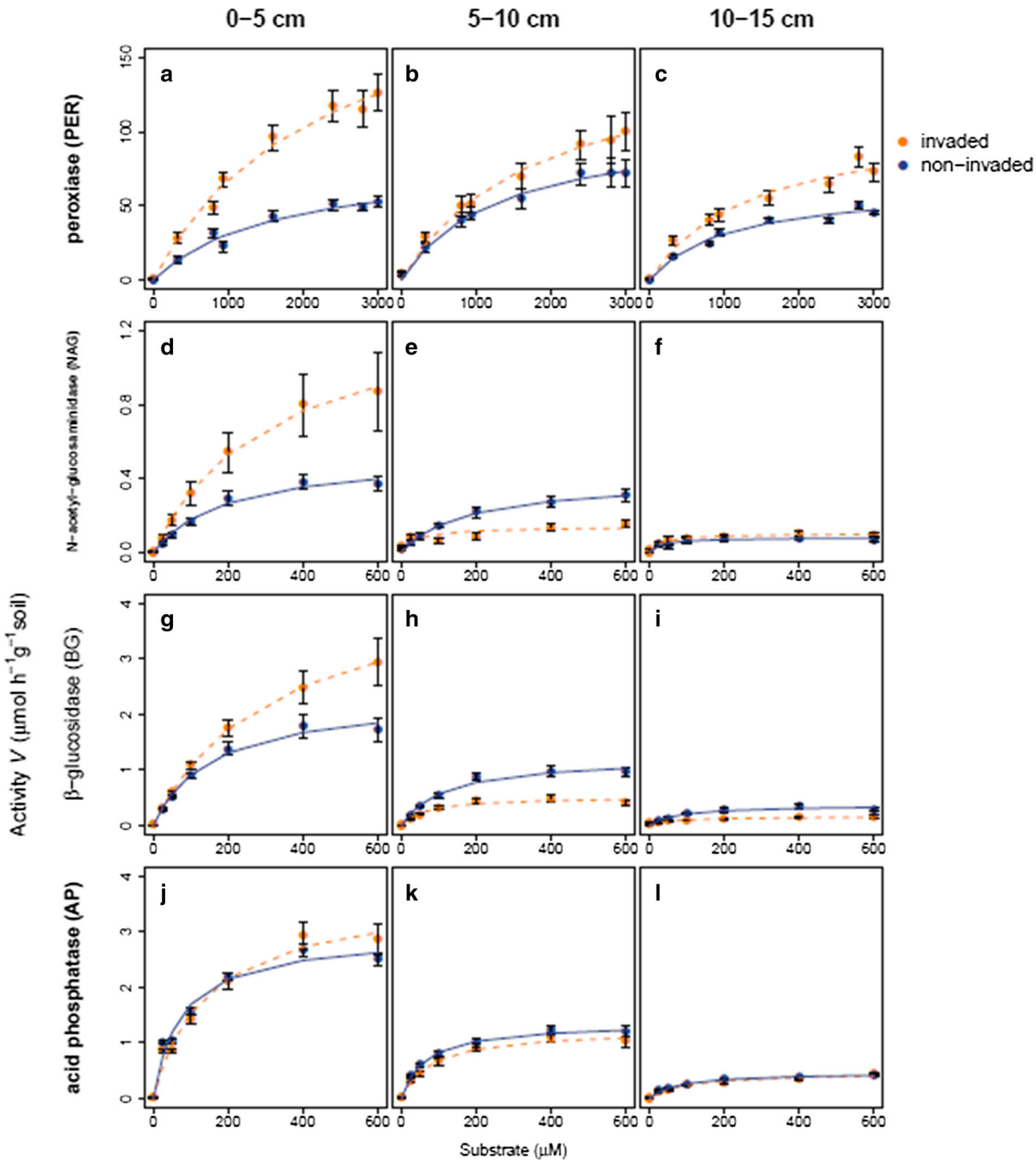
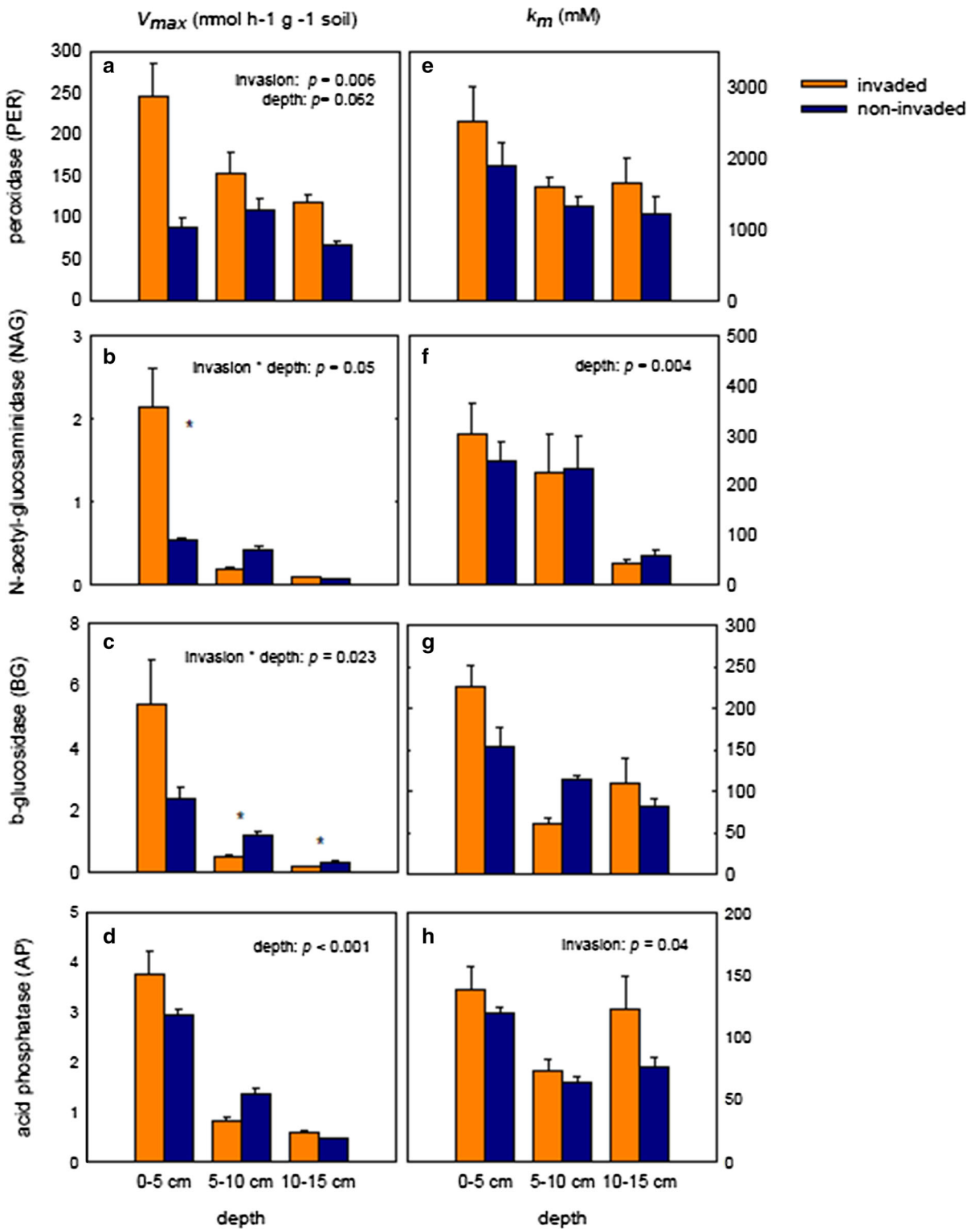


Fig. 5 Enzyme activity *V* as a function of substrate concentration *[S]* under invasion (orange circles, invaded; blue circles, non-invaded) at 0–5, 5–10, and 10–15 cm (n = 6). Peroxidase (**a–c**); *N*-acetyl-glucosaminidase (**d–f**); *β*-glucosidase (**g–i**); acid phosphatase (**j–l**). (Color figure online)

produced different amount of enzymes from those in native grassland, but maintained the production of the same type of isozymes. Microbial community under invasion was likely to maximize the benefit of the relatively abundant substrates and maintained their capacity to capture excess resources through higher production of enzymes. For example, we observed an absolutely (Supplementary Figs. 1, 2) and relatively


V_{max} (nmol h-1 g -1 soil)
k_m (mM)
invaded
non-invaded
peroxidase (PER)
N-acetyl-glucosaminidase (NAG)
b-glucosidase (BG)
acid phosphatase (AP)
a
Invasion: p = 0.006
depth: p= 0.062
e
b
Invasion * depth: p = 0.05
f
depth: p = 0.004
c
Invasion * depth: p = 0.023
g
d
depth: p < 0.001
h
Invasion: p = 0.04
0-5 cm
5-10 cm
10-15 cm
depth

◀**Fig. 6** Michaelis-Menten parameter V_{max} of peroxidase (**a**); *N*-acetyl-glucosaminidase (**b**); *β*-glucosidase (**c**); acid phosphatase (**d**), and k_m of peroxidase (**e**); *N*-acetyl-glucosaminidase (**f**); *β*-glucosidase (**g**); acid phosphatase (**h**) under invaded (orange bars) and the non-invaded soils (blue bars) at 0–5, 5–10, and 10–15 cm (n=6). Significant difference between the invaded and the non-invaded soils at each depth was marked with asterisk. (Color figure online)

(Figs. 2, 3) higher abundance of SOM, lignin, and DOC under invasion and a significant, positive relationship between PER production and lignin content (Table 2). Hence, if microbes had switched their enzyme production from high affinity to low-affinity enzymes when lignin concentration became high under invasion, the net resource gain from lignin decay would have decreased. However, by generating high-affinity enzymes when lignin was abundant under invasion, microbes were able to decay more lignin (high affinity of enzyme + more substrates = more net gain) compared to those in the non-invaded soils (Fig. 5a–c). Such overflow of resources to microbes has been reported under relatively high substrate availability (Russel and Cook 1995; Manzoni et al. 2012; Szenk et al. 2017). Indeed, litter input under *P. cuspidatum* invaded soils was 2.5 times higher than the adjacent, non-invaded soils (Tamura and Tharayil 2014). Microbial overconsumption of substrates partially explains the accelerated nutrient cycling rates at topsoils under *P. cuspidatum* invaded (Dassonville et al. 2007), particularly by the fall season (Tharayil et al. 2013). Moreover, the enzymatic transformation products may persist in the invaded soils (Suseela et al. 2016) that could negatively affect the growth of the native species (Moravcová et al. 2011; Murrell et al. 2011; Dommanget et al. 2014).

Michaelis–Menten kinetics under plant invasion varies with enzymes and depth

Our second hypothesis that the degree to which invasion modifies enzyme kinetics would decrease with increasing soil depth was supported for the V_{max} of NAG. When soil depth increased from 0 to 5 cm to 10–15 cm, the difference in the V_{max} between the invaded and the non-invaded soils reduced by $\sim$ 85% for NAG (Fig. 6b), due to the reductions in the relative chitin content (Fig. 3) and fungal biomass (Fig. 4) along with depth.

Microbial enzyme production was highest at 0–5 cm in both invaded and non-invaded soils (higher V_{max}, Fig. 6), where the contents of SOM, lignin, and chitin were higher than 5–10 cm or 10–15 cm (Fig. 2). Even though we did not test individual microbial population, our results are in line with the evolutionary-economic behavior of microbial resource allocation strategies (Allison et al. 2014). At any point, microbes need to decide how to allocate resources among biomass generation, new enzyme production, respiration, and communication with neighboring microbes. Contrary to resource allocations to other processes (e.g., biomass production, respiration) which can benefit microbes relatively quickly and directly, microbial enzyme production is a rather risky investment of current resources. Once generated, microbial extracellular enzymes should be exuded into the soil matrix and move in a water film to encounter corresponding substrates. Neighboring cheater cells, enzyme-mineral interaction, water and substrate availability can all influence the success of this microbial investment, which can be gauged as a greater acquisition of new resources than the amount of resources used for enzyme generation. As such, microbes would have evolved to generate more enzymes when the corresponding substrates exist in

Table 2 Pearson's correlation coefficient (*r*) and *p* value between substrate and Michaelis–Menten parameters of corresponding enzymes

	PER		NAG		BG		AP	
	V_{max}-lignin	k_m-lignin	V_{max}-chitin	k_m-chitin	V_{max}-cellulose	k_m-cellulose	V_{max}-organic P	k_m-organic P
r	0.45	0.39	0.13	0.01	0.15	0.04	− 0.45	− 0.04
p	0.0061	0.0174	0.4812	0.9401	0.4022	0.8320	0.0071	0.8173

PER peroxidase, *NAG N*-acetyl-glucosaminidase, *BG β*-glucosidase, *AP* acid phosphatase, *P* phosphorus

abundant quantities to increase their likelihood of success (Benner 1989; Allison et al. 2011, 2014; German et al. 2011). In this study, higher relative abundance of lignin and increase in fungal biomass along with increases in PER and NAG generation (higher *[S]* and higher *[E]*in the Eqs. 1 and 2) under invasion at 0–5 cm would have promoted microbial success in resource acquisition.

Conclusions

Our results suggest that the input of relatively recalcitrant substrates by *P. cuspidatum* altered the kinetics of microbial extracellular enzymes. The soils under *P. cuspidatum* had higher lignin and fungal biomass than the adjacent non-invaded soils. We observed a corresponding increase in the V_{max} of PER and NAG in the invaded soils, which degrades lignin and chitin, respectively. The enzymatic transformation of the recalcitrant chemical compounds in *P. cuspidatum* litter further alters the carbon chemistry under *P. cuspidatum* soils. The persistence of these enzymatic transformation products in the invaded soils may negatively affect native species and thus interfere with restoration efforts. The management practices that can effectively alter the soil chemistry and nutrient cycling in the invaded soils - would benefit the native species. Thus, understanding the enzymatic transformation of plant litter and subsequent changes in nutrient and C cycling in the invaded habitats would help to formulate knowledge-based restoration practices.

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