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Canopy herbivory can mediate the influence of plant genotype on soil processes through frass deposition

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Abstract

The ecosystem consequences of intraspecific genetic variation is an emerging field of research that strengthens the link between population and ecosystem ecology. Despite recent advances, it remains unclear under what conditions (abiotic and biotic) plant genetic variation will be important to belowground processes. Forest canopy herbivores can have large influences on soil processes by altering the timing, quantity, and quality of forest floor inputs. We demonstrate that the frass inputs from canopy folivores (forest tent caterpillars and gypsy moths) reflect the intraspecific variation in green leaf chemistry (C:N, condensed tannins) of the aspen clones on which they fed. We then varied the genotype and nutrient availability of aspen and monitored the decomposition of both gypsy moth frass and senesced leaf litter in laboratory microcosms for 63 days. Aspen genotype influenced the short-term, frass-induced soil respiration as well as the longer-term, litter-induced respiration. In addition, aspen genotype interacted with nutrient availability to influence the activity of extracellular enzymes measured at the end of the experiment. These results suggest that in aspen forests, canopy herbivores can mediate the influence of intraspecific variation on ecosystem processes through frass deposition. Intraspecific variation is likely more important to ecosystem functioning than previously thought when trophic interactions are also taken into account. The potential for genetic variation within a single plant species to influence the ecosystem effects of herbivores highlights the importance of understanding how and when genetic variation matters to ecosystem processes.

Keywords: Intraspecific variation; Canopy herbivory; Soil respiration

1. Introduction

Herbivores play important roles in forest ecosystems, influencing plant communities (Mulder et al., 1999; Wardle et al., 2002; Fine et al., 2004) and nutrient cycling both above- and belowground (Pastor and Cohen, 1997; Belovsky and Slade, 2000; Wardle et al., 2002). Although herbivore influences on belowground nutrient cycling have been of considerable interest for decades (Schowalter et al., 1986, 1991; Hunter, 2001), the extent and variability of these impacts remain unclear (Lovett et al., 2002; Bardgett and Wardle, 2003).

Forest herbivores can significantly alter the timing and quality of aboveground inputs to belowground processes (Hunter, 2001; Hartley and Jones, 2004). Even at endemic levels, forest canopy herbivores can have important influences on soil nutrient cycling (Hunter et al., 2003; Stadler et al., 2004; Throop and Lerdau, 2004). For example, during a moderate outbreak of folivores in the southeastern Appalachians, Reynolds et al. (2000) found a three-fold increase in frass, a five-fold increase in soil nitrate availability, and a doubling of stream nitrate exports. These effects are magnified at outbreak levels when complete defoliation can severely alter soil water relations, nutrient cycles, and microbial activity (Lovett and Ruesink, 1995).

Frass deposition is an important, direct mechanism through which canopy herbivores influence belowground processes (Hunter, 2001; Hartley and Jones, 2004; Stadler et al., 2004). Insect frass contains high amounts of nitrogen and is comprised mainly of labile organic material that decomposes quickly (Lovett and Ruesink, 1995), in contrast to the more recalcitrant leaf litter that senesces

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in non-outbreak years. Coprophagous soil arthropods can further increase nutrient release from insect frass and increase soil microbial activity (Zimmer and Topp, 2002). Perturbations to the nitrogen cycle are especially severe because herbivores defoliate leaves before plants resorb nitrogen, as they would under non-outbreak conditions (Lovett et al., 2002). Consequently, herbivores can double the amount of nitrogen transferred from the forest canopy to the soil when compared with leaf litter alone (Hollinger, 1986). Frass is typically thought to increase labile nitrogen inputs, contributing to nitrogen (N) leaching (Swank et al., 1981; Reynolds et al., 2000; Frost and Hunter, 2004). However, in some systems, labile carbon (C) provided by insect frass allows for microbial uptake of N, resulting in decreased N availability for plant uptake (Lovett et al., 2002).

Variation in plant phenotype can have multiple ecosystem level consequences (Whitham et al., 2003). In particular, the chemical variation within a dominant forest canopy species can potentially influence soil processes. Intraspecific variation in secondary metabolites (such as tannins and other phenolics) has been shown to influence leaf litter decomposition and belowground nutrient cycling (Madritch and Hunter, 2002; Madritch et al., 2006). While simple phenolics are often quickly leached from leaf litter and increase soil respiration by acting as carbon substrates, tannins are typically attributed with retarding litter decomposition and soil microbial activity (Horner et al., 1988; Schimel et al., 1996, 1998; Harborne, 1997; Fierer et al., 2001). Variation in secondary metabolites can also influence the distribution of canopy herbivores (Schultz, 1983; Donaldson and Lindroth, 2006), which may result in spatial variation in both the quantity of herbivore-derived frass and the resulting belowground impacts. The *quality* of herbivore-derived frass is also likely to vary according to canopy chemistry. At outbreak levels common to the native forest tent caterpillar (FTC) and introduced gypsy moth (GM), defoliation is often complete regardless of leaf chemistry (Mattson et al., 1991; Donaldson, 2005). The intraspecific chemical variation inherent in green leaves may remain important to soil processes in outbreak years, as frass likely reflects the chemical composition of consumed leaf material.

Plant phenotype determines the ecological role of a species and is controlled by both genotype and the environment. Consequently, investigating the responses of different genotypes in different environments is essential for understanding the relative importance of genotype, environment, and their interactions ($G \times E$) to ecosystem functioning. Here we present the results of experiments designed to investigate: (1) the interactive effects of tree genotype and nutrient availability on insect frass quality, and (2) the effects of host-mediated variation in frass quality on soil microbial activity. Using trembling aspen and GMs as our model system, we compare the microbial activities associated with frass and leaf litter decomposition as influenced by tree genotype and nutrient availability.

2. Methods

2.1. System description

Trembling aspen (*Populus tremuloides*) is the most widespread native tree in North America and among the most genetically diverse known to science (Mitton and Grant, 1996). It is a primary food source for the native FTC (*Malacosoma disstria* Hubner) and the introduced GM (*Lymantria dispar* L.). Populations of both folivores can vary widely among years, with widespread and often complete canopy defoliation of susceptible host species in outbreak years.

The five aspen genotypes used in this study were originally collected from south-central Wisconsin: Dan1 & Dan2 (Dane County, WI), Wau1 & Wau2 (Waushara County, WI), and Sau3 (Sauk County, WI). All five have been identified as distinct genotypes by microsatellite markers. Of 16 loci evaluated, each genotype contained at least four, and up to eight, unique alleles (Cole, 2005).

2.2. Leaf, litter, and frass collection

Foliage for both herbivore species was provided by three-year-old saplings generated via micropropagation techniques (Sellmer et al., 1989; Donaldson and Lindroth, 2006). In 2001, trees were grown in 5 l pots containing a 60:40 mix of torpedo sand and silt-loam field soil. All trees received $4.5\,g\,l^{-1}$ 14–14–14 (N–P–K + micronutrients) Osmocote® 3–4 month slow release fertilizer (Scotts Company, Marysville, Ohio) in 2001 (year 1). In 2002, trees were transferred to 40 l pots containing a 70:30 mix of torpedo sand and silt-loam soil and nutrient treatments were initiated. Trees in the low-nutrient treatment received $0.5\,g\,l^{-1}$ 18–6–12 (N–P–K + micronutrients) Osmocote® 8–9 month slow release fertilizer, while trees in the high-nutrient treatment received $4.5\,g\,l^{-1}$ (Hemming and Lindroth, 1995; Osier and Lindroth, 2001). In spring 2003, fertilizer treatments were repeated except that low-nutrient trees received no fertilizer. For each genotype and nutrient treatment, four replicate trees were allocated to insect feeding trials, while three replicate trees were allocated to fall leaf senescence collection. Green leaf chemistry samples were collected from the set of trees used for insect feeding in the spring of 2003, after leaf expansion and immediately before herbivory treatments. Leaf samples were flash frozen in liquid nitrogen, freeze-dried, and stored at −15 °C. Naturally senesced leaf litter was collected in the fall of 2003 in $1\,cm^2$ mesh netting. Netting was emptied throughout leaf senescence to prevent leaching by intermittent rains. Litter samples were air-dried and stored frozen at −15 °C.

GM egg masses for these experiments were obtained from USDA-APHIS (Otis Air National Guard Base, Massachusetts). Egg masses were surface sterilized for 5 min using a 1.9% solution of commercial bleach containing Tween 80. Larvae were hatched and reared inside

150 × 25 mm rearing cages in a Percival environmental chamber set at 25 °C with a photoperiod of 15:9 (light:dark). FTCs were collected in the second and third stadium from northern Wisconsin in early June of 2003 and reared under the same conditions. GM larvae were fed standard GM wheat germ diet (ICN; Irvine, CA), and FTC larvae were fed field-collected aspen leaves. When the respective larvae reached the mid-fourth stadium, ca. 35 individuals were randomly assigned to rearing cages ($n = 4$) and treatment diets were initiated. Each rearing cage represented one experimental unit (i.e., one replicate of treatment). Potted aspen trees were paired with replicate rearing dishes and randomly selected sprigs of foliage were removed from trees, inserted into waterpiks and added to the respective cages as needed. After two days, i.e., sufficient time for previous diets to be purged, we cleaned the cages and began collecting frass on a daily basis. Collected frass was immediately transferred to clean containers and stored in a −15 °C freezer until further use.

2.3. Microcosm experiment

To test the influence of substrate on microbial activity, we established experimental microcosms using senesced leaf litter and GM frass (FTC produced insufficient amounts of frass). Each microcosm consisted of a 5 × 18 cm cylinder of clear acrylic tubing with a fiberglass mesh bottom leading into a funnel and capped with a rubber seal. All microcosms received 100 g of air-dried silt loam field soil collected from an undisturbed aspen forest in southern Wisconsin. Soil was sieved (2 mm) and well mixed before being placed into microcosms, and allowed to equilibrate for one week at 30 °C and 80–90% RH in a Percival incubator.

Microcosms with litter treatments received 1.3 g of air-dried, chopped (5 mm × 5 mm) litter from each genotype and nutrient treatment in triplicate (5 genotypes × 2 nutrient availability × 3 replicate trees = 30 microcosms). Litter was chopped to prevent individual leaves from completely covering the soil surface in microcosms and impeding subsequent flushes with ddH_2O (below). Frass microcosms received 1 g of dried frass from each genotype and nutrient treatment in quadruplicate (5 genotypes × 2 nutrient availability × 4 replicate trees = 40 microcosms). Amounts of leaf litter and frass were chosen to fall within the range of normal inputs per soil area during normal and outbreak years, respectively. These calculations were based on aspen specific leaf area (Lindroth and Hwang, 1996), aspen leaf area index (Burrows et al., 2002), aspen litterfall values (Raich and Nadelhoffer, 1989; Steele et al., 1997; Davidson et al., 2002), and GM digestion efficiencies (Roth et al., 1998). All microcosms ($n = 70$) were randomized, and incubated at 30 °C and 80–90% RH in a Percival incubator for 60 days.

2.4. Sample analysis

Green leaves, leaf litter, and frass were analyzed for condensed tannin (CT) concentrations using the *n*-butanol method of Porter et al. (1986). Purified aspen tannins (Hagerman and Butler, 1989) served as the standard. Frass and litter carbon and nitrogen levels were determined with a Carlo Erba CNS analyzer. Ammonium concentrations in leachates (below) were determined colorimetrically using assays based on the indophenol blue method modified by Mulvaney (1996) and using sodium dichloroisocyanurate as a hypochlorite source. Nitrate determinations were made by passing leachates through a copper coated cadmium reduction column, followed by subsequent reaction with sulfanilamide and N-1-naphthylethylenediamine dihydrochloride to form an azo dye (Mulvaney, 1996).

General microbial activity was measured via soil respiration using a PP Systems infrared gas analyzer with a chamber adapter modified to securely fit each microcosm. Respiration was measured with decreasing frequency beginning at day 0 and continuing on days 1, 2, 3, 5, 8, 11, 15, 18, 24, 33, 47, and 63. We leached each microcosm on days 0, 30, and 63 with 40 ml of ddH_2O. Prior to starting the experiment, we determined that flushing the microcosms with 40 ml of ddH_2O consistently gave ~15 ml of leachate for analysis.

To further characterize the microbial response to different substrate additions, we measured the activity of six extracellular enzymes in the microcosm soils at the end of the experiment (day 63): cellobiohydrolase and β-glucosidase (involved with the degradation of cellulose), leucine aminopeptidase (involved with the degradation of proteins), phenol oxidase and peroxidase (involved with the degradation of aromatic compounds), and urease (degrades urea). Enzyme assays were based on Sinsabaugh et al. (2000) and Saya-Cork et al. (2002). One to two grams of equivalent dry mass soil from each microcosm were blended in 15 ml 50 mM acetate buffer using steel balls and a modified paint shaker. Four hundred μL of soil extract was then added to 2 ml microcentrifuge tubes in duplicate for each of the six enzyme assays as well as a set for sample blanks. One hundred μl of 5 mM 4-pNP-β-D-cellobioside substrate was used as cellobiohydrolase substrate, 100 μl of 40 mM pNP-β-glucopyranoside was used as β-glucosidase substrate, and 100 μl of 5 mM leucine *p*-nitroanilide was used as leucine aminopeptidase substrate. Soil extracts and substrates were allowed to react for 2 h at 29 °C, then centrifuged at 3000 rev min^{-1} for 10 min to separate soil particles. Supernatant samples were removed and aliquoted in duplicate into 96-well microplates. After receiving 40 μl of 1.5 M NaOH, plates were then read at 410 nm with a spectrophotometer. A *p*-nitrophenol standard curve was used for cellobiohydrolase, β-glucosidase, and leucine aminopeptidase. Phenol oxidase and peroxides activities were estimated in duplicate using 400 μl of soil extract and 100 μl of L-DOPA with and without 40 μl of 0.3% H_2O_2. Soil extracts and substrates were allowed to react for 3 h at 29 °C before centrifuging as above. We used a purified horseradish peroxidase standard curve as a reference and read absorbance at 460 nm. Phenol oxidase activity was

indicated by L-DOPA metabolism in the absence of H_2O_2, while peroxidase activity was measured as the difference between L-DOPA metabolism in the presence and absence of H_2O_2. Urease was measured in duplicate by analyzing soil extract ammonium concentration before and after a 2 h incubation of 400 µl soil extract with 40 µl of 400 mM urea. Ammonium concentrations were determined with the sodium salicylate/sodium dichloroisocyanurate method (above). All activities were converted to µMol substrate h^{-1} g^{-1} soil before statistical analysis.

Excluding the urease assay, commercially available substrates were used to ensure that enzyme assays were standardized and could be compared to other published studies. A limitation of these assays, however, is that the may not reflect actual in situ enzyme activities, rather they describe the relative effects of treatments on enzyme activities in microcosm soils.

2.5. Statistical analysis

We employed repeated measure ANOVAs to test for the effects of time, aspen genotype, soil nutrient availability, substrate type (leaf litter or frass), and their interactions on soil respiration. ANOVAs were used to test for the effects of genotype, nutrient availability, substrate, and their interactions on CT, carbon, and nitrogen concentrations, as well as on soil enzyme activities. Enzyme activities were also analyzed in a complementary fashion by using PC-ORD (v. 4.14) to calculate non-metric multidimensional scaling (NMS) axes, followed by subsequent analysis with MANOVAs to test for treatment interaction effects. Simple correlations were used to correlate green leaf chemistry with frass chemistry, and to compare substrate chemistry with soil respiration. Unless otherwise mentioned, all statistical analyses were performed using SAS JMPIN (v. 4.0.4). Throughout the text, effects with $P<0.05$ are considered significant, effects with $0.05<P<0.1$ are considered marginally significant, and effects with $P>0.1$ are considered not significant.

3. Results

3.1. Genotype and nutrient effects on leaf, litter, and frass chemistry

Aspen genotype and nutrient availability both strongly influenced the chemistry of green leaves, senesced leaf litter, and folivore frass, with nutrient availability having more influence than plant genotype (Table 1, Fig. 1). As expected, low-nutrient treatments produced lower quality leaf material across all aspen genotypes, and this effect was especially apparent in senesced leaf litter. Several gene by environment (G × E) interactions are apparent, with strong genotype × nutrient availability effects on leaf and litter CT concentrations, and on the C:N ratio of frass from both folivore species. In general, FTC frass had lower C:N and CT concentrations when compared with GM frass and was less influenced by nutrient availability than was GM frass.

Table 1
ANOVA results of genotype, nutrient availability, and their interactions on leaf, litter, and frass chemistry

	C:N ratio			Condensed tannin		
	F	D.F.	*P*	*F*	D.F.	*P*
Leaves						
Genotype	4.69	4, 38	0.007	64.39	4, 39	<0.001
Nutrient	353.19	1, 38	<0.001	95.72	1, 39	<0.001
Geno. × Nutr.	2.26	4, 38	0.087	3.71	4, 39	0.014
Litter						
Genotype	5.77	4, 28	0.018	9.41	4, 28	<0.001
Nutrient	292.20	1, 28	<0.001	57.28	1, 28	<0.001
Geno. × Nutr.	2.50	4, 28	0.077	11.13	4, 28	<0.001
FTC frass						
Genotype	13.83	4, 36	<0.001	17.60	4, 38	<0.001
Nutrient	170.97	1, 36	<0.001	0.01	1, 38	*n.s.*
Geno. × Nutr.	6.42	4, 36	<0.001	2.33	4, 38	0.079
GM frass						
Genotype	27.49	4, 37	<0.001	30.52	4, 39	<0.001
Nutrient	242.40	1, 37	<0.001	22.01	1, 39	<0.001
Geno. × Nutr.	17.40	4, 37	<0.001	1.14	4, 39	*n.s.*

Notes: $P>0.1$ indicated as "*n.s.*"

3.2. Leaf chemistry and frass chemistry regressions

Frass chemistry was strongly influenced by green leaf chemistry (Table 2, Fig. 2), demonstrating that herbivore frass reflects the variation of green leaf chemistry. The C:N ratio of frass increased relative to that of leaves, as both herbivores assimilated N from green leaves (Fig. 2). Low-nutrient availability markedly increased all C:N values. The slopes of low-nutrient C:N regressions were roughly twice those of high-nutrient regressions, indicating that both herbivores produced increasingly lower quality frass under low-nutrient availability. Green leaf and frass tannin concentrations followed a 1:1 relationship, with one exception: when aspen trees were grown under low-nutrient conditions, FTC produced frass with less tannin than that of the leaf material consumed (Table 2, Fig. 2).

3.3. Genotype, nutrient availability, and substrate effects on soil microbial activity

Aspen genotype influenced soil respiration across all treatments differently over time (Table 3). Aspen genotype also interacted with substrate type to affect soil respiration differently over time. Most of the genotype-mediated variation in respiration occurred in the first three weeks for litter treatments and in the first week for frass treatments (Table 3, Fig. 3). Leaf litter additions induced higher cummulative respiration than did frass additions over the course of the entire experiment despite a high

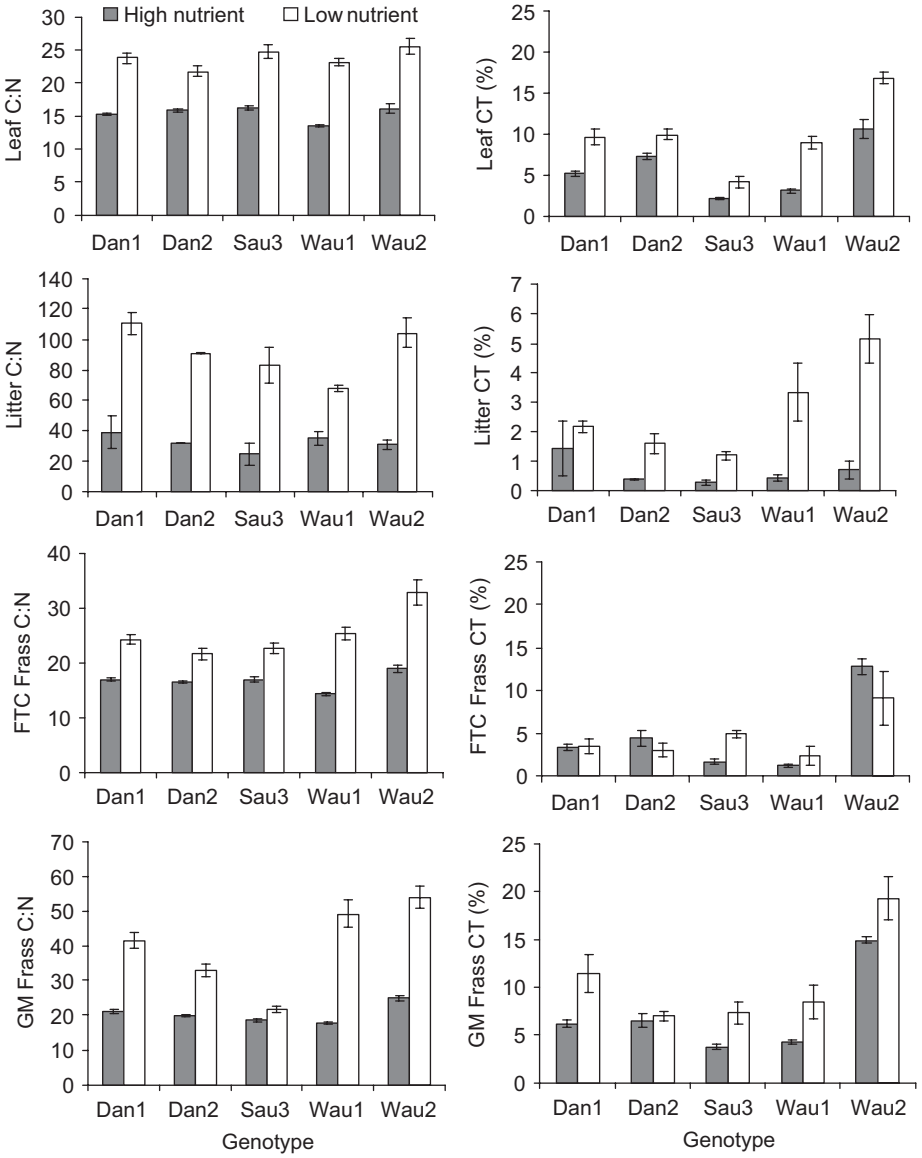

Fig. 1. Genotype and nutrient influences on the chemistry of aspen leaves, litter, and herbivore frass. CT = condensed tannins, FTC = forest tent caterpillar, GM = gypsy moth. Litter $N = 3$, frass $N = 4$. Error bars represent standard error. See Table 1 for ANOVA results.

initial frass-induced respiration spike (Litter $= 720 \pm 72$ g $CO_2\,m^{-2}$, Frass $= 528 \pm 43$ g $CO_2\,m^{-2}$, $F_{1,69} = 6.36$, $P = 0.014$). A significant interaction between aspen genotype and substrate type on soil respiration (Table 3) indicates that insects can mediate the effects of aspen genotypes on soil microbial activity, depending upon whether foliage is allowed to senesce or consumed and deposited as frass.

We expected treatment effects to be due, in part, to genetically mediated variation in substrate chemistry. However, despite the large treatment effects of genotype and substrate on soil respiration, respiration was only weakly related to substrate C:N and CT concentrations ($R^2 = 0.065$, $P = 0.036$ and $R^2 = 0.047$, $P = 0.051$, respectively). Moreover, although tannins are known to be important to belowground nitrogen cycling, the amount of nitrogen leached was unaffected by any treatment and only weakly influenced by the tannin concentration of applied substrates ($R^2 = 0.060$, $P = 0.044$). Tannins can leach quickly from litter and while they typically sorb tightly to soils (Schofield et al., 1998), some may have been lost in the early flushes.

Aspen genotype interacted with nutrient availability and substrate type to influence the soil enzyme activity profile. The activities of several classes of soil enzymes were influenced by the interaction of genotype with nutrient treatment (leucine aminopeptidase $F_{4,69} = 2.59$, $P = 0.048$ and urease $F_{4,69} = 2.27$, $P = 0.075$) and substrate treatment (cellobiohyrdolase $F_{4,69} = 2.58$, $P = 0.048$, β-glucosidase $F_{4,69} = 2.53$, $P = 0.050$, and phenol oxidase $F_{4,69} = 2.42$, $P = 0.061$). NMS analysis distilled the six enzyme activities into two axes, allowing for entire enzyme profiles to be considered together (Fig. 4). When analyzed

in this fashion, aspen genotype interacted with nutrient availability to create distinct enzyme activity profiles ($F_{4,50} = 2.99$, $P = 0.027$). In addition, litter substrate additions created different microbial enzyme profiles than did frass additions ($F_{1,50} = 4.06$, $P = 0.049$).

4. Discussion

Aspen genotype, nutrient availability, canopy herbivory, and their interactions influenced soil microbial activity through frass deposition. The direct influences of aspen genotype on leaf litter decomposition (Madritch et al., 2006) have been attributed to the large variation in green leaf chemistry that can persist through leaf senescence (Lindroth et al., 2002). This study documents that genetic variation within trembling aspen can also influence soil processes through interactions with canopy herbivores. In short, this body of research demonstrates that intraspecific genetic variation in the chemical composition of a common tree species has the potential to influence ecosystem processes through both direct and indirect (herbivore-mediated) effects on organic substrate decomposition.

Table 2
Results of regression analysis for green leaf and herbivore frass chemistry

Frass C:N	R^2	P	N
FTC: C:N = −1.19 + 1.15 (Leaf C:N)	0.81	<0.001	36
High nutrient: C:N = 0.13 + 1.1 (Leaf C:N)	0.65	<0.001	18
Low nutrient: C:N = −23.23 + 2.06 (Leaf C:N)	0.61	<0.001	18
GM: C:N = −17.80 + 2.50 (Leaf C:N)	0.68	<0.001	37
High nutrient: C:N = 2.19 + 1.20 (Leaf C:N)	0.61	<0.001	18
Low nutrient: C:N = −39.88 + 3.42 (Leaf C:N)	0.29	0.017	19
Frass condensed tannin (CT)			
FTC: CT = 0.34 + 0.55 (Leaf CT)	0.34	<0.001	39
High nutrient: CT = −2.33 + 1.22 (Leaf CT)	0.78	<0.001	19
Low nutrient: CT = 0.00 + 0.46 (Leaf CT)	0.29	0.013	20
GM: CT = 1.10 + 1.00 (Leaf CT)	0.73	<0.001	40
High nutrient: CT = 0.81 + 1.12 (Leaf CT)	0.78	<0.001	20
Low nutrient: CT = 0.58 + 1.02 (Leaf CT)	0.66	<0.001	40

Notes: Results are first provided for each insect species across both nutrient treatments, and then separated by nutrient treatments (immediately below).

Aspen forests regularly experience complete defoliation during GM and FTC outbreaks (Mattson et al., 1991; Donaldson, 2005), and such outbreaks change the timing and quality of nutrient inputs into underlying soils (Lovett et al., 2002). Lovett and Ruesink (1995) used microcosms to show that frass stimulates microbial growth by providing a labile carbon source and thereby increasing soil respiration. Likewise, Frost and Hunter (2004) demonstrated that frass additions increase soil microbial biomass in oak mesocosms. Here we show that aspen genotype can influence the magnitude of frass-induced soil respiration. In addition, the interactive effect of plant genotype and nutrient availability on soil enzymes highlights the importance of genetic variation in a heterogeneous environment.

4.1. Frass chemistry reflects leaf chemistry

We hypothesized that genotypic variation in aspen green leaf chemistry would result in genotypic variation in

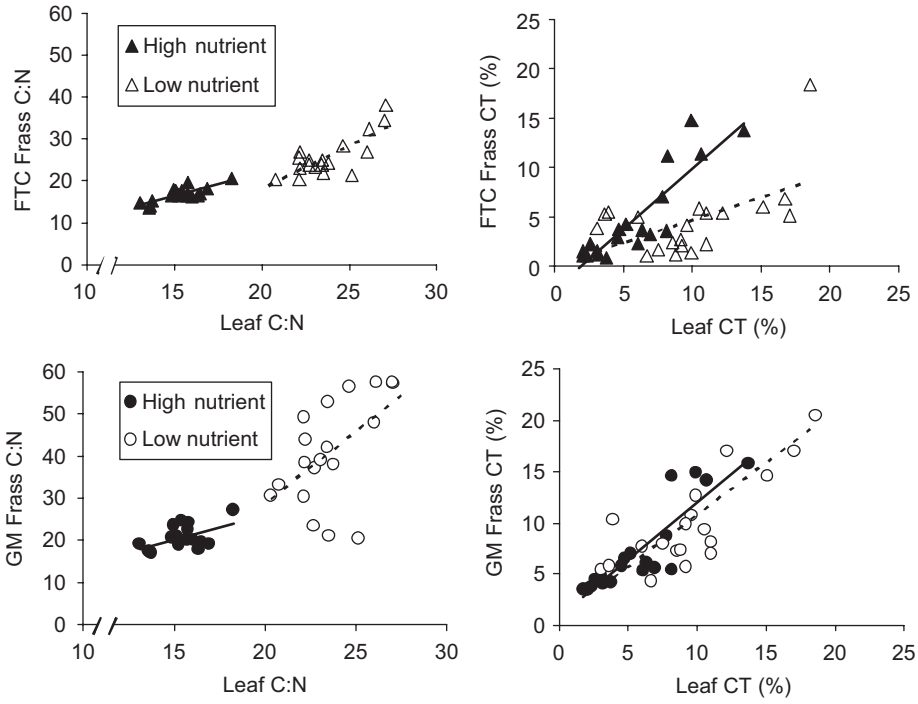

Fig. 2. Regressions of frass chemistry with green leaf chemistry for forest tent caterpillars (FTC) and gypsy moths (GM) across high and low-nutrient availability. Solid and dashed lines represent high and low-nutrient treatment regressions, respectively. See Table 2 for regression results.

Table 3
Repeated measures ANOVA results for genotype, fertility, substrate (litter or frass) and their interaction on respiration over time

Respiration repeated measures	D.F.	*F*	*P*
Genotype	4, 50	1.85	*n.s.*
Nutrient	1, 50	0.26	*n.s.*
Substrate	1, 50	6.69	0.013
Genotype × nutrient	4, 50	1.52	*n.s.*
Genotype × substrate	4, 50	3.52	0.013
Nutrient × substrate	1, 50	2.20	*n.s.*
Genotype × nutrient × substrate	4, 50	0.72	*n.s.*
Time	12, 39	65.08	<0.001
Genotype × time	48, 152.27	1.55	0.023
Nutrient × time	12, 39	6.94	<0.001
Substrate × time	12, 39	28.06	<0.001
Genotype × nutrient × time	48, 152.27	0.76	*n.s.*
Genotype × substrate × time	48, 152.27	1.82	0.003
Nutrient × substrate × time	12, 39	1.52	*n.s.*
Genotype × nutrient × substrate × time	48, 152.27	0.99	*n.s.*

Notes: $P > 0.1$ indicated as "*n.s.*"

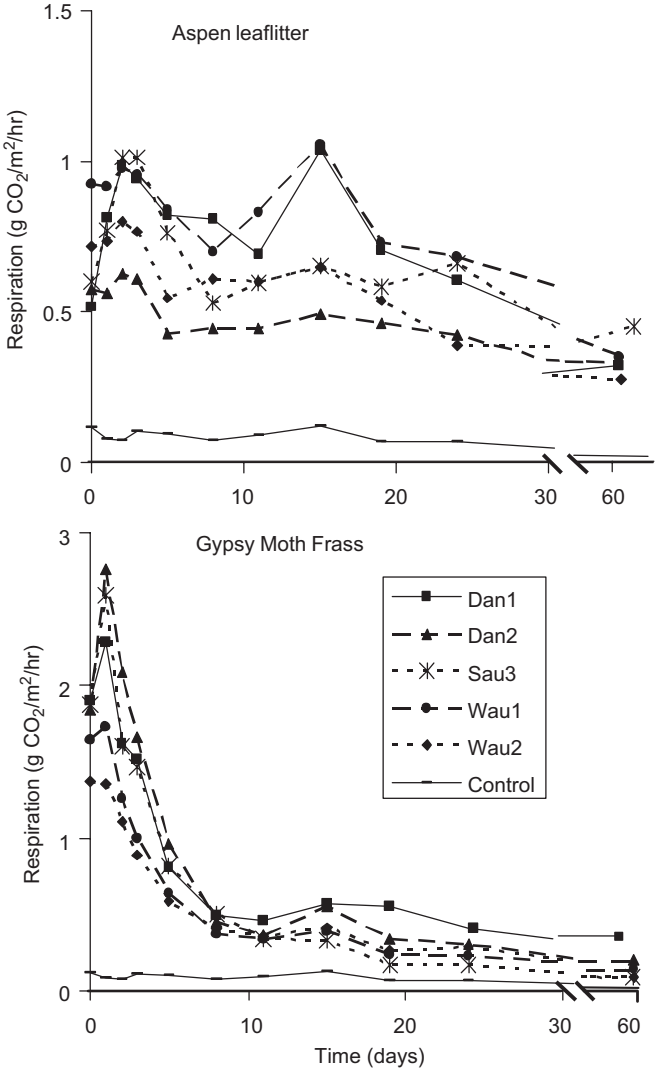

Fig. 3. Early respiration varied by genotype, and by the interaction of genotype with substrate type, over time. See Table 3 for ANOVA results.

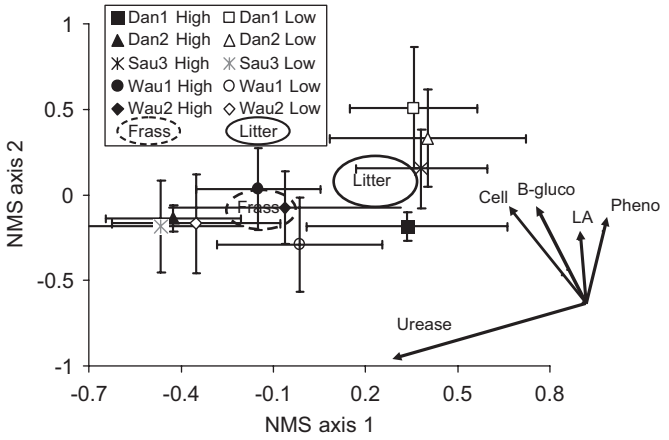

Fig. 4. Plot of the first two axes derived from NMS analysis of six extracellular enzyme activities. Each point represents the average of seven microcosms. Error bars represent standard error for genotype × nutrient treatments. Frass and litter ellipses represent standard errors around the averages of each treatment. The joint plot is offset from the origin for clarity and shows the correlation of enzyme activities with the ordination space. The angle and the length of the vectors indicate the direction and strength of the relationships. Cell = cellobiohydrolase, B-gluco = β-glucosidase, LA = leucine aminopeptidase, Pheno = phenol oxidase. Peroxidase activity is not shown on the joint plot because it did not explain variance among microcosms.

herbivore frass. Although frass chemistry reflected green leaf chemistry (Table 2, Fig. 2), the two herbivore species responded differently to the same green leaf material. GM larvae produced frass with a higher C:N ratio than did FTC larvae, especially when fed foliage from low-nutrient trees. This difference suggests that GM are more efficient than FTC at extracting protein from aspen diets, with consequent effects on frass quality. As expected, aspen grown under low-nutrient availability had higher concentrations of CTs than did aspen gown under high nutrients (Donaldson and Lindroth, 2006). This difference was mirrored in CT concentrations in GM frass, but not in FTC frass (Figs. 1 and 2). Interestingly, FTC reared on low-nutrient aspen did not produce frass with elevated concentrations of tannins. Frass produced by FTC reared on low-nutrient aspen contained less tannin than did the leaf material upon which they fed. The reason for this disparity between FTC and GM is unknown, but could be due to binding of tannins to other gut or frass constituents and reduced efficiency of tannin extraction from FTC frass. Alternatively, tannins may degrade while passing through FTC guts. Detritivores are known to degrade polyphenolics in consumed leaf litter (Zimmer, 1999; Zimmer et al., 2005), but we have no direct evidence that forest canopy herbivores metabolize polyphenolics. Regardless of the mechanism, our data demonstrate that different canopy herbivores respond differently to leaf chemistry, so herbivore-mediated impacts on organic substrate decomposition are likely to be species-specific.

4.2. Frass and litter treatment effects

During outbreak years in natural forests, spring-feeding herbivores such as GM and FTC defoliate and deposit frass early in the growing season. Leaves then reflush, often with altered leaf chemistry (Lyytikainen, 1994; Donaldson, 2005; Stevens and Lindroth, 2005). These leaves persist until senescence, upon which they return a second influx of nutrients to belowground food webs. Our study was designed to simulate outbreak frass, but not subsequent reflushed leaf litter deposition. Hence, our microcosm study did not fully represent "outbreak" and "non-outbreak" years for natural forest ecosystems. However, we can make important comparisons between the litter and frass decomposition. First, cumulative soil respiration was higher for the leaf litter addition treatments despite high initial respiration peaks in the frass addition treatments (Fig. 3). Litter treatments induced more cumulative soil respiration probably because litter treatments received more mass (and hence carbon) than did frass treatments. This was done to mimic consumption by herbivores, as herbivores necessarily consume a portion of the green leaf material that would otherwise senesce and fall as leaf litter. Presumably in natural forests, defoliated stands would experience a frass-induced respiration peak, followed by an additional litter-induced respiration peak after the growing season ended. Second, we found interactive effects of genotype and substrate (frass or litter) treatments on soil respiration (Table 3). A genotype by substrate interaction indicates that aspen genotypes can influence soil respiration differently depending upon herbivory; genotypes that induce high microbial activity during canopy defoliation events may not necessarily also induce high activity during leaf litter senescence. Based on our soil respiration data, herbivores have the potential to mediate the influence of intraspecific variation on ecosystem processes through frass deposition.

The suite of extracellular enzymes found in soils represents the functional activity of the microbial community, and may be more relevant to ecosystem functioning than is microbial taxonomic diversity (Caldwell, 2005). Extracellular enzyme activity increases when target nutrients are present in complex forms (Allison and Vitousek, 2005), such as the N contained in folivore frass (Lovett et al., 2002). Consequently, the frass and litter difference along the urease vector (Fig. 4) is likely due to the relatively high amount of N available in frass compared to litter ($1.84 \pm 0.11\%$ versus $1.06 \pm 0.11\%$, respectively), and the likelihood that frass N occurs in a complex form. The interactive effect of genotype and nutrient availability on extracellular enzyme activities further demonstrates $G \times E$ effects of aspen genotype on the soil microbial community.

The labile carbon source provided by frass additions likely resulted in net immobilization, and thereby conservation, of nitrogen (Lovett and Ruesink, 1995). Consequently we found no genotype or substrate treatment effects on N leached from our microcosms, as presumably most N was immobilized in the soil microbial biomass or other organic complexes (e.g., tannin–protein complexes).

4.3. Limitations

While microcosms are useful for understanding simple processes, all microcosm studies have inherent limitations because they do not fully mimic natural conditions (Carpenter, 1996; Verhoef, 1996). We used microcosms in a first step toward understanding how genetic variation in host plants can influence the belowground impacts of herbivore frass. In such a simple system, we have limited the biotic and abiotic factors that normally influence ecosystem processes in natural forests. For instance, we have not considered the activity of invertebrate detritivores that play important roles in the detrital pathway (Coleman and Crossley, 1996). When Zimmer and Topp (2002) included coprophagous detritivores in a similar experiment, both the period of duration and the amount of microbial respiration induced by frass increased in the presence of detritivores. Furthermore, frass deposition is only one of many mechanisms by which forest herbivores influence belowground nutrient cycling (Hunter, 2001). Nonetheless, our results demonstrate the potential for plant genotype to indirectly influence soil microbial activity through herbivore frass deposition.

4.4. Broader implications

In addition to depositing large amounts of frass, forest herbivores can influence belowground processes by altering the chemistry of host trees (Hunter, 2001; Bardgett and Wardle, 2003). Schweitzer et al. (2005a, b) found that genetic variation in a *Populus* hybrid system can interact with canopy herbivory to influence leaf litter decomposition. Tree genotype influenced herbivore-mediated changes in litter decomposition rates, which occurred presumably due to herbivore-induced changes in leaf and litter chemistry. Data presented here demonstrate similar potential for genotype and genotype $\times$ herbivore influences on belowground processes in a single species system through frass deposition.

Whitham et al. (2003) employed the concept of "extended phenotypes" (the effects of genes at levels higher than the population) to argue for a community genetics approach that integrates genetic and ecosystem science. Several studies have shown that tree genotype can influence belowground processes through litter decomposition (Madritch and Hunter, 2002; Schweitzer et al., 2004, 2005a, b; Madritch et al., 2006). Here, we demonstrate that the effects of aspen genotype extend through another trophic level (herbivores) to influence belowground processes. This represents an interspecific indirect genetic effect (sensu Shuster et al., 2006) in which the genetic variation in one species alters the ecosystem level impacts of another. In addition, we report $G \times E$ effects of aspen genotype and nutrient availability on the soil community. $G \times E$ interactions are ecologically and evolutionarily

important (Via and Lande, 1985) and can maintain genetic diversity in natural populations if different genes are selected for in different environments (Gillespie and Turelli, 1989). We have shown similar G × E effects on aspen leaf litter decomposition (Madritch et al., 2006) and such interactions may be important for maintaining genetic diversity within widespread plant species such as aspen. However, we have no evidence of feedback that would select for a particular aspen genotype. Genetic variation in aspen causes variation in microbial community activity, yet it is unknown whether the variation in microbial community will select for one genotype at the expense of another. Absent this latter connection of higher order effects to allele frequency, some researchers (e.g., Biernaskie and Tyerman, 2005) have questioned whether an extended phenotype perspective is warranted. Selective feedbacks are not necessary for extended phenotypes to exist, but the community genetics implications are limited without evidence of feedbacks that alter allele frequency. Nonetheless, understanding the influence of genetic variation on ecosystem processes is a worthwhile goal in its own right, and is also an essential first step in testing the viability of a community genetics approach toward ecosystem science.

5. Conclusions

We conclude that the chemistry of frass produced by forest canopy herbivores reflects the genetic variation of consumed green leaf material. Furthermore, canopy herbivores can potentially mediate the effects of intraspecific genetic variation on belowground microbial processes. Aspen genotypes influenced microbial decomposers through their interaction with folivores and resource availability. Genetic variation has the potential to influence ecosystem functioning directly through leaf litter decomposition, and indirectly through trophic interactions such as herbivory. In natural systems, direct and indirect effects occur in concert, and it is likely that the combined effects of intraspecific genetic variation on ecosystem processes are more important than previously thought. As diversity within species declines due to anthropogenic forces, it becomes increasingly important to elucidate the consequences of genetic variation and trophic interactions for ecosystem functioning.

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