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Phenolic-rich leaf carbon fractions differentially influence microbial respiration and plant growth

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Abstract Phenolics can reduce soil nutrient availability, either indirectly by stimulating microbial nitrogen (N) immobilization or directly by enhancing physical protection within soil. Phenolic-rich plants may therefore negatively affect neighboring plant growth by restricting the N supply. We used a slow-growing, phenolic-rich alpine forb, *Acomastylis rossii*, to test the hypothesis that phenolic-rich carbon (C) fractions stimulate microbial population growth and reduce plant growth. We generated low-molecular-weight (LMW) fractions, tannin fractions, and total soluble C fractions from *A. rossii* and measured their effects on soil respiration and growth of *Deschampsia caespitosa*, a fast-growing, co-dominant grass. Fraction effects fell into two distinct categories: (1) fractions did not increase soil respiration and killed *D. caespitosa* plants, or (2) fractions stimulated soil respiration and reduced plant growth and plant N concentration while simultaneously inhibiting root growth. The LMW phenolic-rich fractions increased soil respiration and reduced plant growth more than tannins. These results suggest that phenolic compounds can inhibit root growth directly as well as indirectly affect growth by reducing pools of plant available N by stimulating soil microbes. Both mechanisms illustrate how below-ground phenolic effects may influence the growth of neighboring plants. We also examined patterns of foliar phenolic concentrations among populations of *A. rossii* across a natural productivity gradient (productivity was used as a proxy for competition intensity). Concentrations of some LMW phenolics increased significantly in more productive sites where *A. rossii* is a competitive equal with the faster growing *D. caespitosa*. Taken together, our results contribute important information to the growing body of evidence indicating that the quality of C moving from plants to soils can have significant effects on neighboring plant performance, potentially associated with phytoxic effects, and indirect effects on soil biogeochemistry.

Keywords Allelopathy · Low-molecular-weight phenolics · Mineral nutrition · Phenolics · Plant growth · Plant secondary compounds · Plant–soil interactions · Soil nitrogen · Tannins

Introduction

In terrestrial ecosystems, phenolic compounds can comprise a significant portion of the carbon (C) pool that moves from plants to soil because many plant species contain high concentrations of phenolic compounds in complex mixtures (Bate-Smith 1962; Levin 1971). Phenolics may therefore be an important determinant of plant C quality and significantly affect decomposition and soil nutrient availability (Horner et al. 1988; Schimel et al. 1996; Kraus et al. 2003). Despite immense phenolic structural diversity, these compounds can be grouped into two general categories with differing functional attributes: low-molecular-weight (LMW) phenolics that do not precipitate proteins, and higher molecular weight condensed and hydrolyzable tannins that are defined by their ability to precipitate proteins (Hagerman and Butler 1989). Low-molecular-weight phenolics and tannins have traditionally been studied as

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defenses against herbivores and pathogens (McArthur et al. 1993; Walton 1997), as mediators of pollination (Thompson et al. 1972), as UV screens and anti-oxidants (Feild et al. 2001; Close and McArthur 2002), and as allelopathic agents (Callaway and Aschehoug 2000; Bais et al. 2003). However, more recent studies show that both LMW phenolics and tannins are also important in shaping a plant's soil nutrient environment (Kuiters 1990; Schimel et al. 1996; Fierer et al. 2001; Hättenschwiler et al. 2003; Bowman et al. 2004; Kraus et al. 2004).

Phenolics may influence the plant nutrient environment differently from other sources of C (e.g., sugars and cellulose) due to their chemical reactivity. For example, sugars and cellulose affect soil C and nitrogen (N) dynamics primarily as a function of their lability and their subsequent ability to stimulate microbial growth and the immobilization of N. In contrast, phenolics can shape the soil nutrient environment via multiple pathways. Mechanistically, LMW phenolics reduce soil nutrient availability to plants similarly to sugars and cellulose—by stimulating soil respiration and the immobilization of N in microbial biomass (Schimel et al. 1996, 1998; Fierer et al. 2001). Tannins may also act via this pathway to a limited degree (Kraus et al. 2004). However, because tannins bind to proteins and N-rich soil organic matter, tannins may also lower gross soil N mineralization and nitrification rates by binding to microbial enzymes or enzyme substrates (Northup et al. 1998; Fierer et al. 2001; Kraus et al. 2004). Plant leaves or litter with high concentrations of phenolics could therefore indirectly inhibit the growth of neighboring plants by reducing plant available N and affecting the soil resource environment via at least two distinct pathways. Phenolic-mediated reductions in plant available N would be particularly noticeable in N-limited plant communities (Bowman et al. 2004). However, it is not well understood how important labile LMW phenolics are in soils relative to tannins in terms of their influence on soil C and N cycling and, in particular, few studies have examined the effects of LMW phenolic-rich C on the growth of neighboring plants (but see Nilsson et al. 2000).

We have examined the effects of phenolic-rich C fractions on soil microbial activity and plant growth within the context of alpine moist-meadow tundra plant communities on Niwot Ridge, Colorado. Alpine moist meadows present a useful model system for testing the effects of phenolic-rich C on plant growth for two reasons: (1) primary production is N-limited (Bowman et al. 1995); (2) alpine plant communities contain numerous, abundant species of phenolic-rich forbs (Dearing 2001). Moreover, phenolic-rich species influence localized biogeochemical cycling of C and N and appear to reduce plant available forms of soil N (Steltzer and Bowman 1998). In alpine moist meadows of the Southern Rocky Mountains, the slow-growing, N-conservative forb *Acomastylis rossii* is a competitive equal with the fast-growing grass *Deschampsia caespitosa* (Suding et al. 2004). *Acomastylis rossii* litter contains $20.7 \pm 1.3\%$ dry weight (DW) phenolics [gallic acid equivalents (GAE); Steltzer and Bowman 1998], while *D. caespitosa* litter contains 1% GAE DW or less (Bowman et al. 2004). Previous experiments have shown that *A. rossii* litter stimulates soil respiration and the immobilization of N in microbial biomass and that *A. rossii* litter reduces *D. caespitosa* growth (Bowman et al. 2004). These data are consistent with the hypothesis that *A. rossii* litter—and, potentially, litter phenolics—provide a C substrate for soil microbes, stimulate microbial N immobilization, and thus reduce *D. caespitosa* growth by restricting the N supply.

In the study reported here, we characterized the chemical composition of two LMW C fractions and a tannin fraction extracted from fresh *A. rossii* leaves and leaf litter. By means of a laboratory soil incubation experiment and a greenhouse experiment, we then tested the hypothesis that labile phenolic-rich fractions from *A. rossii* tissues stimulate soil microbes and reduce *D. caespitosa* growth. We predicted that soils amended with phenolic-rich *A. rossii* leaf and litter fractions would show elevated soil respiration relative to control soils. Furthermore, we predicted *D. caespitosa* grown in soils amended with *A. rossii* fractions would show: (1) decreased biomass and plant N accumulation compared to plants grown in control soils; and (2) increased root:shoot biomass ratios compared to control plants. Combining the predicted outcomes from these two experiments, we anticipated there would be a significant negative relationship between plant growth and soil respiration and that labile LMW C fractions would have a greater impact on soil respiration and plant growth than tannin fractions.

The hypothesized effects of phenolics on soil nutrient availability and neighboring plant growth should help the slow-growing *A. rossii* persist in the presence of faster growing neighbors in the competitive environment of alpine moist meadows (Suding et al. 2004). To investigate whether increasing foliar phenolic concentrations may be correlated with competitive intensity in the field, we took advantage of the established positive relationship between above-ground standing crop and competition intensity (Twolan-Strutt and Keddy 1996; Foster 1999, 2000) and the fact that *A. rossii* is abundant across a gradient of above-ground standing crop (Bowman and Fisk 2001). We predicted that *A. rossii* plants growing in communities with high above-ground standing crop would show increased concentrations of those phenolics that are most inhibitory to other species' growth compared to *A. rossii* plants growing in communities with low above-ground standing crop.

Materials and methods

We tested our predictions by amending field-collected native, moist-meadow soils with phenolic-rich LMW C and tannin fractions from fresh *A. rossii* leaves and recently senesced *A. rossii* leaf litter. We measured the microbial respiration response in one experiment and the *D. caespitosa* growth response in a second experiment. We generated extracts from fresh *A. rossii* leaves because previous studies have used fresh leaves as a phenolic-rich C source (Schimel et al. 1996, 1998; Fierer et al. 2001), and fresh leaves have been shown to transport soluble C (including phenolics) directly to soil via canopy throughfall (McClaugherty 1983). We also generated extracts from *A. rossii* litter, as litter may represent a larger and more readily available pool of phenolic-rich C from an ecosystem perspective. We analyzed *A. rossii* foliar phenolic concentrations across a natural competition intensity gradient to determine whether patterns of foliar phenolic concentrations in the field are consistent with the hypothesized effects of phenolics on soil respiration and neighboring plant growth that we tested in the laboratory.

Collection of plants and soils for laboratory and greenhouse experiments

Deschampsia caespitosa plants and alpine moist-meadow soils (top 15 cm) were collected in September 2004 from Niwot Ridge, Colorado (40°03′N, 105°35′W, 3500 m a.s.l., a National Science Foundation Long Term Ecological Research site). After harvesting, *D. caespitosa* plants were transplanted and maintained in an actively growing state in an environmental chamber at the University of Colorado [photoperiod: light (15 h/15°C):dark (9 h/10°C)] for several months before being used in experiments. Immediately after harvesting, soils were stored in the dark at 4°C for 8 months prior to being sieved (2-mm mesh) and homogenized for experimental use. A comparison of soil respiration rates before and after an 8-month storage-period indicated labile soil C was significantly reduced by this treatment, thereby ensuring that amending soils with *A. rossii* C fractions would result in detectable soil microbial responses (unpublished data).

Generation of *A. rossii* soluble C fractions

Total soluble carbon (TSC) fractions were generated from fresh, mature *A. rossii* leaves and recently senesced leaf litter that were harvested from Niwot Ridge in July 2003 and September 2003, respectively. Fresh leaves were immediately placed on dry-ice while still in the field, transported to the laboratory, and freeze-dried. Leaf litter was kept at ambient temperature and air-dried. Dried leaves were finely ground in a mortar and pestle with liquid N_2, and ground leaves were extracted with hexane to remove waxes and oils. Ground leaves were then extracted four times sequentially with 70% aqueous acetone, and the acetone was removed by rotary evaporation. The resulting aqueous solution was centrifuged to remove precipitate (20 min, 3200 *g*). To produce TSC fractions suitable for application to soils, this aqueous solution was freeze-dried onto purified silica gel (240- to 400-mesh, Whatman #4790-010) for use as a soil amendment (as described in Schimel et al. 1996). Silica-sorbed TSC fractions were then mixed thoroughly with soil. The use of silica gel ensured a complete recovery of extracts and allowed compounds that are only sparingly soluble in water to be evenly distributed throughout the soil.

To obtain LMW C and tannin fractions, we chemically separated TSC fractions from *A. rossii* fresh leaves and leaf litter using methods described in Hagerman (2002). We obtained the following fractions: (1) a relatively hydrophobic ethyl acetate-soluble fraction containing some LMW compounds (the LMW HP fraction); (2) a water-soluble fraction comprised mostly of LMW compounds (the LMW WS fraction); (3) a hydrophilic tannin fraction comprised of one chemical species of ellagitannin. Characterization of the ellagitannin is described in Meier et al. (2008). The relative hydrophilicity of each C fraction was determined by qualitatively assessing the extent to which a known mass of each fraction was soluble in ethyl acetate, methanol, 50% aqueous methanol, and water (Table 1). All soluble C fractions were sorbed to silica gel prior to application to native, sieved, alpine moist-meadow soils, as described above.

To ensure that the amounts of C added to soils were within a biologically relevant range, we first calculated annual above- and below-ground litter inputs per square meter for monospecific stands of *A. rossii* in alpine moist meadows (Steltzer and Bowman 1998; Bowman and Fisk 2001). We then used the area of the soil microcosms (soil respiration experiment) or the plant pots (plant growth experiment) to calculate the mass of each *A. rossii* fraction added per gram DW soil (Table 2). Although the total amount of C added is what is expected due to litter inputs, it was added in one dose rather than gradually as might occur under field conditions. Fractions were added to soil corresponding to their relative abundance in leaves (Tables 1, 2), allowing us to more accurately assess the potential impact of each fraction on respiration and plant growth in the field. As such, soils did not receive equal amounts of C among treatments (Table 2). To measure the difference in C quantity added to soil, we freeze-dried and weighed the amount of each fraction that was generated from a standard quantity of leaf tissue (1 g), using the same

Table 1 Chemical analysis of soluble carbon (C) fractions isolated from *Acomastylis rossii* fresh leaves and leaf litter

	Soluble C fractions					
	A. rossii fresh leaves			*A. rossii* leaf litter		
	LMW HP	LMW WS	Tannin	LMW HP	LMW WS	Tannin
General						
Total fraction mass	63.0 ± 0.9	241.4 ± 0.9	121.8 ± 0.8	99.6 ± 0.7	135.5 ± 1.7	114.5 ± 2.0
Total fraction C	31.4 ± 0.4	99.1 ± 0.4	58.1 ± 0.4	47.7 ± 0.4	57.1 ± 0.7	54.4 ± 1.0
Total fraction N[a]	0.048 ± 0.001	0.625 ± 0.002	0.102 ± 0.001	0.109 ± 0.001	0.411 ± 0.005	0.117 ± 0.002
C:N	655 ± 24	159 ± 4	569 ± 15	437 ± 3	139 ± 2	466 ± 5
Solubility (H_2O)	+	+++	+++	+	+++	+++
Phenolics[b]						
Anthocyanin	–	–	–	–	0.880 ± 0.051(1)	–
Coumarin	–	5.56 ± 0.03 (1)	–	–	4.95 ± 0.11(2)	–
Ellagitannin	64.8 ± 1.0 (13–15)	3.82 ± 0.13 (1)	114 ± 2 (1)	58.2 ± 1.0 (6)	19.1 ± 0.7(9)	77.8 ± 1.4 (1)
Flav Gly	1.86 ± 0.07 (4)	12.2 ± 0.1 (6)	–	14.7 ± 0.3 (4)	6.71 ± 0.21(6–7)	–
Gallotannin	4.14 ± 0.11 (4)	1.25 ± 0.02 (2)	–	1.26 ± 0.05 (2)	–	–
Phen Acid	0.801 ± 0.056 (5)	1.39 ± 0.01 (4)	–	10.6 ± 0.3 (6–7)	3.52 ± 0.11(5)	–
Total phenolics[c]	71.7 ± 1.2 (26–28)	24.2 ± 0.2 (14)	114 ± 2 (1)	84.7 ± 1.0 (18–19)	35.1 ± 0.7 (23–24)	77.8 ± 1.4 (1)
Total phenolic C	35.8 ± 0.6	12.1 ± 0.1	56.9 ± 1.1	42.4 ± 0.5	17.6 ± 0.3	38.9 ± 0.7
Sugars						
Cyclitols	–	10.5 ± 0.2	–	–	1.56 ± 0.03	–
Glucose	–	18.6 ± 0.3	–	–	9.64 ± 0.13	–
Fructose	–	16.1 ± 0.2	–	–	5.87 ± 0.09	–
Sucrose	–	57.5 ± 0.9	–	–	1.53 ± 0.02	–
Total sugars	–	103 ± 1	–	–	18.6 ± 0.2	–
Total sugar C	–	42.3 ± 0.4	–	–	7.47 ± 0.06	–

Values are means ± 1 SE ($n = 3$). Units, where appropriate, are mg g^{-1} dry weight leaf

LMW HP, Low-molecular-weight hydrophobic C fraction; LMW WS, low-molecular-weight water-soluble C fraction; Flav Gly, flavonoid glycosides; Phen Acid, phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids)

[a] Values represent only N found within C fractions, not total leaf/litter N. Values are low as the fractionation procedure was optimized to exclude N-containing compounds

[b] For phenolic entries, the values in parenthesis indicate the number of unique compounds identified within a given phenolic class

[c] Values for "total phenolics" may exceed the total fraction mass due to differences in extinction coefficients between the individual compounds that were detected and the external standard that was used

extraction and fractionation procedures described above ($n = 3$ per fraction). The mass of each fraction (mg g^{-1} leaf) was determined gravimetrically, and mass C in each fraction (mg C g^{-1} leaf) was calculated using %C measurements from a Shimadzu elemental analyzer (%N was also recorded, Table 1). Mass C added to soil for each fraction was then calculated as:

$$\text{mg C g}^{-1}\text{ dry weight soil} = \left[\begin{array}{r}\text{leaf mass (mg)}\\ \text{extracted}\end{array}\right]\left[\frac{\text{fraction mass(mg)}}{\text{leaf mass (mg)}}\right][\text{fraction C content (\%)}][\text{soil mass (g)}]^{-1}$$

In this way, we accounted for both the amount of C and the type of C (fraction identity) added to soils.

Chemical analysis of LMW C and tannin fractions

To characterize the chemical composition of *A. rossii* fractions from both tissue types (fresh leaves and leaf litter), we analyzed fractions (LMW HP, LMW WS, and tannin fractions; $n = 3$ per fraction) for phenolic constituents using high-pressure liquid chromatography (Table 1). The LMW phenolics and tannins were quantified and tentatively grouped into major phenolic classes by comparing retention times and spectra (200–500 nm) with those of external standards. Chromatographic conditions and standards for phenolic analyses are described in Meier et al. (2008). The concentrations of tannins within the fractions, when expressed on a milligram per gram DW leaf basis (Table 1), were approximately 100 mg g^{-1} leaf lower than those of leaf extracts that had not been fractionated

Table 2 Mass and basic composition of *A. rossii* soluble C fractions added to soil for soil respiration and plant growth experiments

Fraction	Mass	Total fraction C	Phenolic C[a]	Sugar C
Fresh leaves				
LMW HP	1.53 ± 0.02	0.765 ± 0.010	0.873 ± 0.014	–
LMW WS	5.88 ± 0.02	2.41 ± 0.01	0.295 ± 0.002	1.03 ± 0.01
Tannin	2.97 ± 0.02	1.41 ± 0.01	1.39 ± 0.03	–
TSC	10.38 ± 0.04	4.59 ± 0.02	2.55 ± 0.03	1.03 ± 0.01
Leaf litter				
LMW HP	2.40 ± 0.02	1.15 ± 0.01	1.02 ± 0.01	–
LMW WS	3.26 ± 0.04	1.38 ± 0.02	0.424 ± 0.008	0.180 ± 0.002
Tannin	2.76 ± 0.05	1.31 ± 0.02	0.936 ± 0.017	–
TSC	8.42 ± 0.07	3.83 ± 0.03	2.38 ± 0.02	0.180 ± 0.002

Values are means ± 1 SE ($n = 3$) and indicate the amount of each fraction added to soil (mg g^{-1} dry weight soil)

TSC, Total soluble C fraction

[a] Values for "total phenolics C" may exceed "total fraction C" due to difference in extinction coefficients between the individual compounds that were detected and the external standard that was used

(unpublished data), suggesting 30–35% of the total tannins were irreversibly bound to the Sephadex LH-20 (Amersham Pharmacia Biotech, Piscataway, NJ) column used for the fractionation procedure. Cyclitols, glucose, fructose, and sucrose were quantified using high-performance anion-exchange chromatography–pulsed amperomatric detection (PAD), as described by Moore et al. (1997).

Soil respiration experiment

Low-molecular-weight HP, LMW WS, and tannin fractions (all $n = 4$) and TSC fractions ($n = 8$) from fresh *A. rossii* leaves and leaf litter as well as purified silica gel controls ($n = 8$) were thoroughly mixed with field-collected moist-meadow soils (50 g DW) in 120-ml specimen cups. Quantities of fractions per gram DW soil are listed in Table 2. Following C amendment, soils were maintained at 50% gravimetric moisture on a weekly basis with distilled water and were kept at 8°C (growing season mean; Bowman et al. 2004) in a growth chamber for 4 weeks. Soil respiration rates were recorded daily with a LI-COR 6200 gas exchange system (LI-COR, Lincoln, NB), and the total amount of CO_2 respired for each treatment was calculated by plotting CO_2 respiration rate versus time and integrating the area under the curve (PrismGraph ver 4.0; GraphPad Software, La Jolla, CA). Carbon substrate use efficiencies were calculated for TSC fractions by dividing the total C–CO_2 respired by the total C added.

Because soils received different amounts of C depending on which fraction was used (Table 2), a parallel soil incubation experiment was performed to assess the influence of fraction C quantity versus fraction C identity on soil respiration. A soil respiration response curve was generated for the LMW HP, LMW WS, and tannin fractions by amending soils with the amount of each fraction that would enter moist-meadow soils on an annual basis (Table 2) as well as a doubling and a halving of the amount we expect would naturally enter soils ($n = 4$ per treatment group). For the LMW HP, LMW WS, and tannin treatments, we then plotted total C–CO_2 respired versus total C added for the three levels of C addition. Microbial C use efficiency was then calculated for each fraction as the slope from a simple linear regression of these data.

Plant growth experiment

Deschampsia caespitosa plants were separated into units of three to four interconnected tillers weighing between 0.4 and 1.4 g fresh weight (FW) each. Dead roots and leaves were removed, and the remaining live leaves were trimmed to 1 cm in length; the plants ($n = 10$ per treatment) were then placed into 6.6 cm × 12-cm cylindrical pots. The pots contained control soils (amended with purified silica gel) or soils amended with *A. rossii* TSC, LMW HP, LMW WS or tannin fractions (121.7 g DW soil per replicate; fraction quantities per gram dry DW are listed in Table 2). Ten unplanted *D. caespitosa* plants were used to calculate the fresh weight to dry weight ratio, and the initial average shoot and root mass as a percentage of total plant mass (plants were oven-dried at 70°C for 48 h). We used these data, in combination with the initial phytometer fresh weight, to calculate leaf and root biomass accumulation for each plant.

Experimental plants were kept in a research greenhouse at the University of Colorado with air temperatures between 8–15°C and a natural light environment, beginning in May 2005. Twice weekly, plants were watered with tap water just to field capacity to minimize water, and thus

A. rossii C amendments, from leaching from the pots. After 10 weeks, *D. caespitosa* plants were harvested, the soil was carefully removed from the roots by gently washing in tap water, and the plants were oven-dried at 70°C for 48 h. The final root and leaf dry masses were recorded, and tissues were analyzed for %N with a Shimadzu elemental analyzer (Shimadzu, Kyoto, Japan).

Because significant plant mortality occurred when soils were amended with LMW HP fractions, we tested whether residual ethyl acetate in these fractions may have been toxic to *D. caespitosa*. A treatment was prepared with ethyl acetate and silica gel, which was added to sieved alpine moist-meadow soil along with a purified silica gel-only control. *Deschampsia caespitosa* was planted in pots as described above ($n = 8$ for each treatment), and plants were grown in a growth chamber in which temperatures varied between 8 and 15°C, and lighting was maintained on a 16/8-h (day/night) cycle. Plants were watered just to field capacity twice weekly with tap water for 10 weeks and were then harvested and weighed as described above.

Analysis of *A. rossii* phenolics across a productivity gradient

To address whether increasing competitive intensity was correlated with foliar phenolic concentrations in the field, we analyzed fresh *A. rossii* leaves and recently senesced leaf litter harvested across a productivity gradient, used as a proxy for competitive intensity (Twolan-Strutt and Keddy 1996; Foster 1999, 2000). Above-ground standing crop of all plant species was quantified in six sites across the gradient in early August at the height of the growing season. All standing live plant tissue from a representative 20 cm × 20 cm square ($n = 8$ per site) was clipped, dried at 70°C for 48 h, weighed, and the mass was recorded to the nearest 0.01 g.

Fresh leaves were harvested from *A. rossii* populations across the gradient in July 2002 (mature leaves only), and recently senesced leaf litter was harvested from the same *A. rossii* populations in September 2002. Fresh *A. rossii* leaves and leaf litter from each site ($n = 4$ replicates per site) were analyzed for phenolics, and each replicate was a composite of leaves harvested from five to six adjacent plants. Fresh leaves were immediately placed on dry-ice while still in the field, transported to the laboratory, and freeze-dried. Leaf litter was kept at ambient temperature and air-dried. The LMW HP, LMW WS, and tannin fractions were then generated for each replicate as described above. Phenolics were quantified in each fraction using Folin–Ciocalteau reagent standardized to gallic acid equivalents (Waterman and Mole 1994).

Statistical analyses

Prior to analysis, residuals were tested for normality with quantile–quantile plots or with the Shapiro–Wilk test. Homogeneity of variance was assessed either by plotting fitted values versus residuals or with Levene's test, and data were log transformed as necessary to meet the assumptions of parametric statistics. Multiple comparisons among means were performed on untransformed data following analysis of variance (ANOVA) with the Tukey HSD test. For the plant growth experiment, treatment groups in which phytometers died were not included in statistical analyses (LMW HP treatments from fresh *A. rossii* leaves and leaf litter). An analysis of co-variance (ANCOVA) was used to determine whether total CO_2 respired from each treatment was a significant predictor of plant growth and leaf N concentration, with plant initial dry weight as the co-variate. Linear regressions were performed on untransformed and log-transformed data using both first order polynomials, and non-linear models where appropriate. Akaike's Information Criterion was used to determine whether linear or non-linear models were more appropriate for the data. Analyses were performed using R ver. 2.5.1 ® Corporation, Vienna, Austria) and PrismGraph ver. 4.0.

Results

Characterization of *A. rossii* C fractions

High-pressure liquid chromatography (HPLC) analysis confirmed that the *A. rossii* LMW HP, LMW WS, and tannin fractions we generated had unique chemical profiles. The three fractions were dissimilar from each other in terms of the number, types, and relative abundance of the compounds we detected (Table 1). The tannin fractions were comprised of only one type of ellagitannin that is abundant in *A. rossii* tissues. The LMW HP and LMW WS fractions from both fresh leaves and leaf litter consisted of numerous compounds representing all of the phenolic classes we quantified (Table 1), and the LMW HP fractions contained small amounts of several ellagitannins that were not detected in the tannin fraction (data not shown). Using the fractionation procedure we employed, it was not possible to generate LMW phenolic fractions that did not contain the ellagitannin we detected in the tannin fraction (Table 1).

We used HPLC to tentatively identify which LMW phenolics were present in our experimental fractions. We then calculated the abundance of phenolics in each fraction to determine whether the composition of the LMW and tannin fractions were dominated by phenolics on a

percentage mass basis. The LMW HP fractions from fresh leaves and leaf litter were mostly phenolic on a mass basis (113.8 ± 1.0% for fresh leaves and 85.0 ± 0.9% for leaf litter; Table 1). The tannin fractions were primarily phenolic on a mass basis as well (93.5 ± 1.7% for fresh leaves and 67.9 ± 0.2% for leaf litter; Table 1). However, the LMW WS fractions from fresh leaves and leaf litter were only 10.0 ± 0.1 and 25.9 ± 0.4% phenolics by mass, respectively, and these fractions contained 42.6 ± 0.6 and 13.7 ± 0.1% sugars by mass, respectively (Table 1). The LMW WS fractions also had higher N concentrations and lower C:N ratios than did the LMW HP and tannin fractions (Table 1). These data indicate that, in addition to the sugars and phenolics we detected, the LMW WS fractions contained significant concentrations of unidentified water soluble leaf metabolites, possibly amino acids (Table 1).

Effects of *A. rossii* fractions on soil respiration

On average, C amendments from fresh *A. rossii* leaves increased soil respiration more than treatments generated from leaf litter ($t = 3.92$, $P < 0.001$; Fig. 1). Total soluble C (TSC) fractions from fresh *A. rossii* leaves and leaf litter were readily mineralized by soil microbes and increased soil respiration by 497 and 205% relative to controls, respectively (Fig. 1).

The fractionation procedure we employed allowed insight into which compounds were likely responsible for the soil respiration response. Soil microbes did not mineralize LMW HP fractions from either fresh *A. rossii* leaves or leaf litter (Fig. 1). The tannin fractions were more stimulatory than the LMW HP fractions and increased soil respiration by 46% (fresh leaves) and 49% (leaf litter) relative to control soils (Fig. 1), although this increase was not statistically significant for soils amended with tannins from fresh *A. rossii* leaves. In contrast, the LMW WS fractions stimulated soil microbes and increased soil respiration by 319% (fresh leaves) and 128% (leaf litter) relative to control soils and showed the highest C use efficiencies of the fractions we tested (Fig. 1).

When the TSC fractions were further fractionated and applied to soil, the soil respiration response to both fresh leaf (data not shown) and leaf litter fractions (Fig. 2) depended strongly on fraction C quantity (fresh leaves: $F_{1,30} = 1{,}350$, $P < 0.0001$; litter: $F_{1,30} = 136.8$, $P < 0.0001$), fraction identity (fresh leaves: $F_{2,30} = 233.8$, $P < 0.0001$; litter: $F_{2,30} = 1{,}159$, $P < 0.0001$), and the interaction between these two terms (fresh leaves: $F_{2,30} = 53.6$, $P < 0.0001$; litter: $F_{2,30} = 8.88$, $P < 0.001$). Similar patterns were obtained for fractions from fresh *A. rossii* leaves and leaf litter. For soils amended with fractions from fresh *A. rossii* leaves, fraction identity explained 23.9% and C quantity explained 69.1% of the variation in the respiration response. For soils amended with leaf litter fractions, fraction identity explained 92.6% and C quantity explained 5.5% of the variation in the respiration response.

High levels of respiration from the LMW WS treatments were associated with the elevated sugar content of these fractions (Table 1), but sugar content alone could not account for the observed levels of soil respiration. For the LMW WS fraction from fresh leaves, approximately 1673 μg C g^{-1} soil was respired after respiration from control soil was subtracted (Fig. 1). Assuming complete mineralization of sugars, total sugar-C in this treatment (approx. 1030 μg g^{-1} soil; Table 2) could account for 61.5% of the total C-CO_2 respired. Similarly, phenolic-C in the LMW WS fraction (approx. 295 μg g^{-1} soil; Table 2) could account for an additional 17.6% of total C respired.

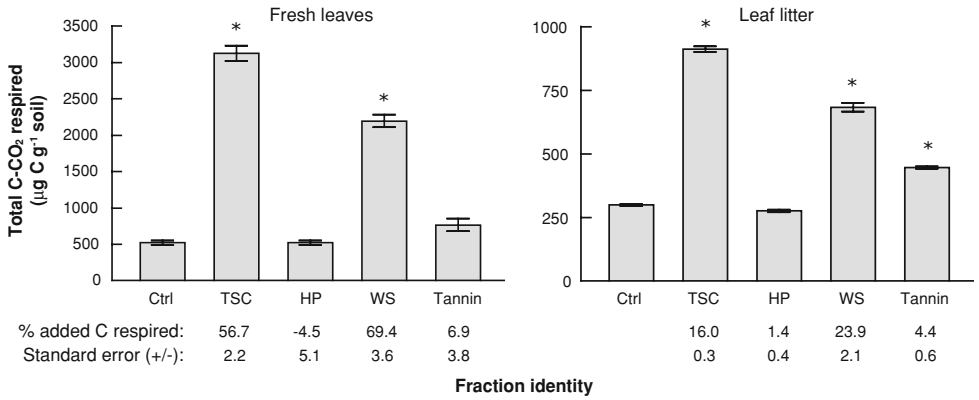

Fig. 1 Soil respiration response to soluble carbon (C) fractions generated from fresh *Acomastylis rossii* leaves and leaf litter. For both panels: *Ctrl* Silica gel control, *TSC* total soluble carbon, *HP* low-molecular-weight hydrophobic fraction, *WS* low-molecular-weight water-soluble fraction, *Tannin* water-soluble ellagitannin fraction. For all treatments, means are ±1 SE ($n = 8$ for Ctrl and TSC treatments, $n = 4$ for all other treatments). *Asterisks* indicate treatments significantly different from the control, $P < 0.0001$ Tukey HSD test. *Numbers* under each treatment indicate the mean C use efficiency (±1 SE) for each treatment (% of added treatment C that was respired as C–CO_2)

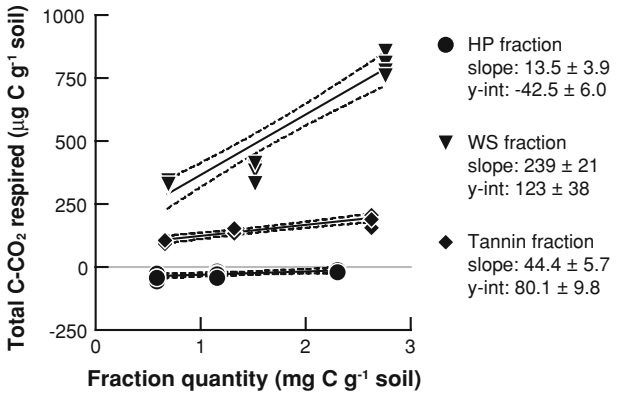

Fig. 2 Soil respiration as a function of both the quantity of *A. rossii* soluble C fractions added and fraction identity. Abbreviations are the same as those used in Fig. 1. The *middle set of points* on each *curve* corresponds to the amount of each fraction that would enter soils on an annual basis given our estimates of moist-meadow primary productivity. The other two levels of C amendment represent a doubling and a halving, respectively, of the amount we expect could naturally enter soils. The *slope* of the regression line is a measure of the C use efficiency for each fraction. *Dashed lines* are the 95% confidence intervals of the regression lines. $n = 4$ for each treatment group

However, soil microbes clearly mineralized other sources of C that were not identified, since sugar and phenolic-C could account for 79.1% (at most) of the total C-CO_2 respired. For the LMW WS fraction from leaf litter, approximately 384 μg C g^{-1} soil was respired after respiration from control soil was subtracted (Fig. 1). For this treatment, sugar-C and phenolic-C could account for all of the C-CO_2 respired (again assuming 100% mineralization). However, sugars and phenolics were probably not mineralized with 100% efficiency, and it is therefore likely that unidentified compounds (which account for 60% of the fraction mass) also contributed to the mineralization response.

Effects of C treatments on plant growth

Total soluble C fractions from both fresh leaves and leaf litter inhibited *D. caespitosa* shoot growth relative to controls by 35.0 and 22.4%, respectively. In addition, TSC fractions reduced *D. caespitosa* root growth by 34.7% (fresh leaves) and 32.2% (leaf litter) relative to controls (Fig. 3). *Deschampsia caespitosa* plants grown in soils amended with *A. rossii* LMW HP, LMW WS, and tannin fractions responded in two distinct ways. First, some plants growing in soils amended with LMW HP fractions were killed within the first 2 weeks of the experiment (80 and 20% mortality for fresh leaf and litter fractions, respectively). Plants that survived in the LMW HP treatments grew very little over the 10 weeks of the study period compared to control plants (Fig. 3). Mortality was not observed for plants grown in TSC amended soils, which contained the same quantity of LMW HP compounds, albeit in the presence of the LMW WS compounds and tannins. Biomass measurements of *D. caespitosa* plants grown in soils amended with ethyl acetate-treated silica gel and an unamended silica gel control showed that residual ethyl acetate sorbed to the silica gel did not affect *D. caespitosa* shoot or root growth (data not shown). Phytotoxic effects from the LMW HP fractions were therefore derived from *A. rossii* leaf/litter chemistry and not residual ethyl acetate.

The second *D. caespitosa* response to *A. rossii* fractions was a decrease in shoot and root growth. The degree of growth reduction depended strongly on fraction identity (Fig. 3). Plants grown with the LMW WS fractions showed significantly reduced shoot and root biomass relative to controls (Fig. 3). Tannins from fresh *A. rossii* leaves inhibited root growth but did not affect shoot growth, and tannins from leaf litter had no effect on *D. caespitosa* shoot or root growth (Fig. 3). In addition, for those soil amendments that did not cause *D. caespitosa* mortality, there was a significant negative correlation between fraction effects

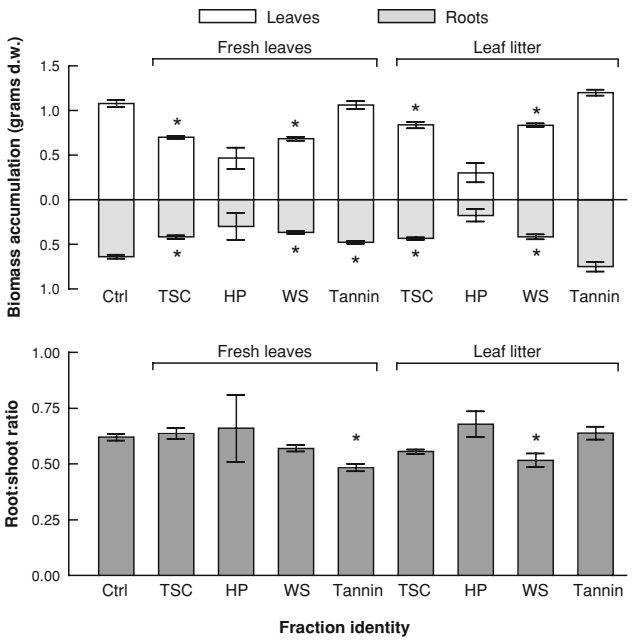

Fig. 3 Growth response of *Deschampsia caespitosa* plants to soluble C fractions generated from fresh *A. rossii* leaves or leaf litter. Biomass accumulation over the course of 10 weeks is shown for *D. caespitosa* phytometer shoots and roots, and root to shoot ratio is shown for plants harvested at week 10. Treatments and abbreviations are identical to those in Fig. 1. For all treatments except HP, means are ±1 SE ($n = 10$). For both HP treatments, phytometer mortality occurred, and means were calculated only for surviving plants ($n = 2$ and 8 for HP fresh leaf and litter treatments, respectively). The LMW HP results were not included in statistical analyses but are shown here for comparison. *Asterisks* indicate treatments significantly different from the control, $P < 0.05$ Tukey HSD test

on soil respiration and the reduction in *D. caespitosa* biomass relative to control plants (slope = −0.16 ± 0.02, $R^2 = 0.67$, $P < 0.0001$; Fig. 4). Across all soil amendments, soil respiration combined with initial plant biomass explained 78% of the reduction in *D. caespitosa* growth relative to control plants ($F_{2,56} = 101.3$, $P < 0.0001$). Increasing soil respiration was also correlated with a reduction in leaf N concentration relative to control plants (slope = −0.032 ± 0.014, $R^2 = 0.12$, t = −2.30, $P < 0.05$). However, soil respiration was not correlated with changes in *D. caespitosa* root N concentration (slope = 0.014 ± 0.013, $R^2 = 0.01$, $t = 1.15$, $P > 0.25$).

In contrast to our initial predictions, soils amended with *A. rossii* fractions did not cause increases in *D. caespitosa* root:shoot ratios. For six of the eight soil C amendments, *D. caespitosa* root:shoot ratios were not significantly different from those of the control plants (Fig. 3). However, relative to the control treatment, the tannin fraction from fresh leaves reduced the root:shoot ratio by 21.9%, and the LMW WS fraction from leaf litter reduced the root:shoot ratio by 16.6% (Fig. 3).

Acomastylis rossii phenolics across a productivity gradient

Concentrations of total phenolics in field-collected fresh leaves and leaf litter were not correlated with standing crop (Fig. 5). However, concentrations of phenolics in the LMW WS fractions were positively correlated with the standing crop (Fig. 5) and increased by a factor of approximately 2.5 as the standing crop increased to its maximal level. Concentrations of LMW WS phenolics were highest in the most productive communities in which *A. rossii* was a competitive equal with *D. caespitosa*.

Patterns in LMW HP and tannin phenolic concentrations were less clear across the gradient. As standing crop increased, *A. rossii* fresh leaves showed variable concentrations of LMW HP compounds, ranging from 3.5 to 6.25% of the dry weight (GAE). Overall, LMW HP phenolic concentrations decreased slightly as standing crop increased (slope = −0.007 ± 0.002, $R^2 = 0.32$, $P < 0.005$). Concentrations of tannins in fresh leaves ranged between 9.0 and 16.3% of dry weight (GAE) but did not change with increasing standing crop ($R^2 = 0.08$, $P = 0.19$).

For *A. rossii* leaf litter, concentrations of LMW HP phenolics ranged between 3.6 and 5.75% of dry weight (GAE), and concentrations were not correlated with standing crop ($R^2 = 0.10$, $P = 0.13$). Leaf litter tannin concentrations ranged between 9.8 and 14.3% of dry weight (GAE), and concentrations declined slightly as

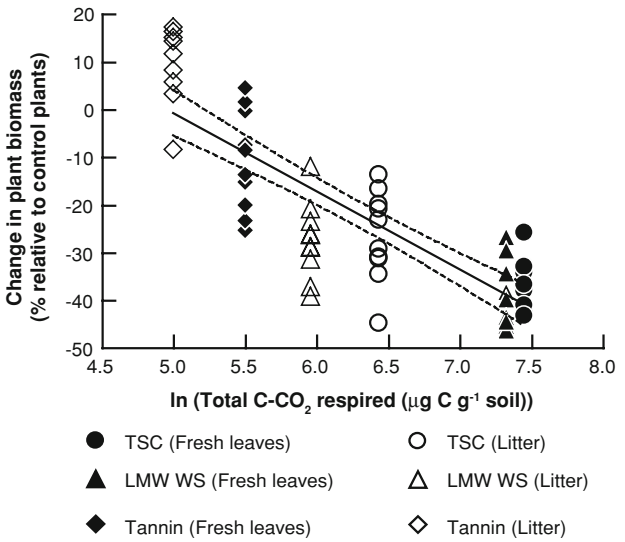

Fig. 4 Correlation between *D. caespitosa* biomass response (% change relative to control plants) and the natural log of soil respiration. Soil respiration data are from the soil respiration experiment (Fig. 1), and the *D. caespitosa* biomass response is from the plant growth experiment (Fig. 3). Respiration from the control soils has been subtracted from each treatment group. *Dashed lines* 95% confidence intervals of the regression line, *filled symbols* plants growing in soils amended with extracts/fractions from fresh *A. rossii* leaves, *open symbols* plants growing in soils amended with extracts/fractions from *A. rossii* leaf litter ($n = 10$ plants per treatment group)

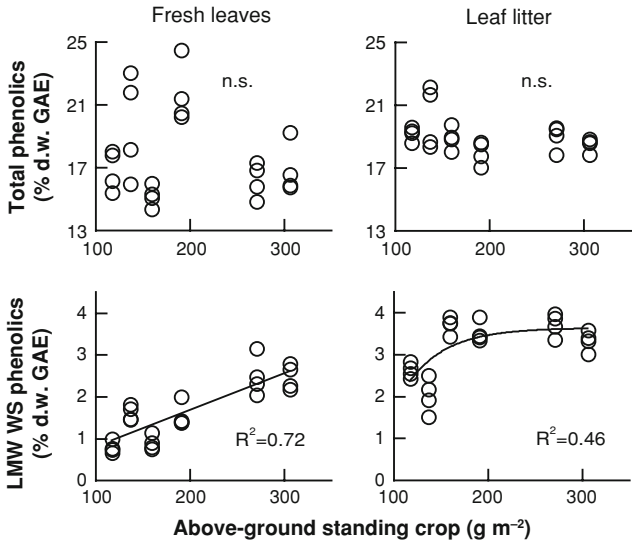

Fig. 5 Phenolic content of *A. rossii* leaves as a function of above-ground standing crop. Above-ground standing crop is a proxy for competition intensity in these plant communities. Fresh *A. rossii* leaves were harvested in July 2002, and freshly senesced *A. rossii* litter was harvested in September 2002. Total phenolic concentrations within total soluble C extracts were standardized to gallic acid equivalents (*GAE*). *LMW WS phenolics* Low-molecular-weight water soluble phenolics (GAE). The LMW WS fractions from *A. rossii* fresh leaves and litter contain sugars (Table 1), but this figure shows only how phenolic quantities within this fraction responded to changes in above-ground standing crop; we did not analyze how other fraction components (sugars, etc.) may have responded

standing crop increased (slope = -0.008 ± 0.003, $R^2 = 0.24$, $P < 0.05$).

Discussion

We used soil incubation and plant growth experiments to test the hypothesis that phenolic-rich C fractions from *A. rossii* stimulated soil microbes and indirectly reduced *D. caespitosa* growth by influencing plant N supply. We found that the effects of phenolic-rich *A. rossii* C fractions on soil respiration and *D. caespitosa* growth fell into two distinct categories: (1) LMW HP fractions were not mineralized by soil microbes, and *D. caespitosa* plants grown in soils amended with LMW HP fractions experienced significant mortality; (2) all other phenolic-rich fractions stimulated soil respiration and reduced *D. caespitosa* growth to varying degrees. Moreover, for those fractions that stimulated soil respiration and did not kill plants, increasing soil respiration was strongly correlated with reduced *D. caespitosa* growth (Fig. 4). We also found that concentrations of some LMW phenolics were higher in leaves of *A. rossii* plants growing in more productive sites, where competitive intensity is likely greater, than in low productivity sites, where competitive intensity is lower. Taken together, these results support the idea that phenolic-rich C fractions simultaneously reduce the growth of neighboring plants via a phytotoxic mechanism as well as by microbial immobilization of N, as we had hypothesized.

Phytotoxic effects of soluble C fractions

Our results provide two lines of evidence consistent with *A. rossii* phenolics reducing *D. caespitosa* growth by a phytotoxic mechanism. First, LMW HP fractions killed *D. caespitosa* plants within the first 2 weeks of the plant growth experiment (Fig. 3). Second, we initially predicted that fractions stimulating high levels of microbial respiration would also be associated with increases in *D. caespitosa* root:shoot ratios, based on experiments in which *D. caespitosa* was grown with *A. rossii* litter (Bowman et al. 2004), and experiments where *D. caespitosa* was grown with reduced soil nutrient concentrations (Bowman and Bilbrough 2001). In contrast to our predictions, reductions in *D. caespitosa* plant growth were not accompanied by higher root:shoot ratios. Even plants grown with the LMW WS fractions did not increase their allocation to roots compared to control plants (Fig. 3), and these fractions contained high levels of sugars (which should not inhibit root growth) and relatively few phenolics (Table 1).

This lack of a root:shoot ratio response and the observed decrease in root:shoot ratios suggest that even low levels of LMW phenolics and tannins may directly inhibit *D. caespitosa* root growth. These results are similar to those reported by Orwin et al. (2006), who showed that mixtures of commercially available compounds containing gallic acid and tannic acid frequently inhibited plant shoot and root growth. Experiments with *Deschampsia flexuosa* have also shown that cinnamic acid effectively inhibits root growth (Kuiters 1990). In addition, LMW phenolics from *Kalmia angustifolia* have been shown to inhibit root growth of *Picea mariana* seedlings (Ren and Mallik 2006). The phytotoxic effects of phenolics on root growth may be derived from these compounds' ability to perturb root cell lipid membranes (Kuiters 1990). Reductions in root growth and perturbed root function represent one mechanism by which phenolic-rich C fractions from *A. rossii* could reduce both *D. caespitosa* shoot growth and tissue N concentration.

Effects of total soluble C mixtures compared to individual fractions

The effects of the LMW HP fractions on soil respiration and plant growth were striking in comparison with those of the other fractions. However, it is notable that the clear phytotoxic effects of the LMW HP fractions were lacking when plants were grown with the TSC fractions. The TSC fractions contained identical quantities of LMW HP compounds and did not kill *D. caespitosa* plants, while the LMW HP fractions by themselves resulted in significant mortality.

There are two non-mutually exclusive potential explanations for why recalcitrant compounds in the LMW HP fraction killed plants in isolation but not in the context of TSC fractions. First, the mixture of labile and recalcitrant compounds in the TSC fractions may have resulted in a microbial priming effect, leading to a greater consumption of LMW HP fractions than occurred in isolation. Consistent with this suggestion, total C-CO_2 respired from the TSC amendments was greater than the sum of the C-CO_2 respired from the individual fractions (Fig. 1), indicating that recalcitrant compounds in the LMW HP or tannin fractions were mineralized in the context of the TSC mixture. Several studies have shown that the mineralization of otherwise recalcitrant C substrates can be accelerated by the addition of simple organic compounds (Hamer and Marschner 2005; Brant et al. 2006) and that the mineralization of substrate mixtures can be more thorough than what is observed with single compounds (Orwin et al. 2006). It is therefore possible that toxic compounds in the LMW HP fractions were consumed when mixed with other compounds in the TSC fractions and that microbial activity may have limited the toxicity of the TSC fractions to the experimental plants.

A second possibility is that soil microorganisms may have been unable to metabolize toxic compounds in the

LMW HP fractions due to nutrient limitation. Blum and Shafer (1988) reported that microbial use of phenolic acids as substrates can be constrained when populations of microorganisms are nutrient deficient. In our experiments, TSC fractions contained more N than LMW HP fractions (Table 1), which may have facilitated mineralization of recalcitrant compounds in the TSC fractions. Furthermore, in a separate experiment, we found that the addition of dilute nutrients stimulated microbial mineralization of LMW HP fractions but did not affect mineralization from soils amended with any of the other fractions (C.L. Meier, unpublished data). Together, these data suggest that without sufficient N to stimulate microbial mineralization, phytotoxic LMW HP phenolics may have persisted in soil, subsequently killing *D. caespitosa* plants.

Differential effects of C fractions on soil respiration and plant growth

Among the fractions that did not kill *D. caespitosa* plants, LMW WS fractions stimulated soil respiration more efficiently than tannins (Figs. 1, 2), which is consistent with previous observations (Fierer et al. 2001; Kraus et al. 2004). The LMW WS fractions also reduced plant growth more than tannin fractions (Fig. 3), and there was a significant negative correlation between increasing soil respiration and *D. caespitosa* biomass accumulation (Fig. 4). In addition, there was a significant negative correlation between increasing soil respiration and *D. caespitosa* leaf N concentration. Previous work in our laboratory established that *A. rossii* litter stimulates soil respiration, increases the production of microbial biomass C and N, and inhibits *D. caespitosa* growth, likely due to microbially mediated reductions in plant available N (Bowman et al. 2004). Consistent with this previous study and our initial predictions, our results support the concept that labile, phenolic-rich C treatments reduced *D. caespitosa* growth via a second indirect mechanism: by stimulating microbial activity and indirectly reducing pools of plant available N.

Our data do not address this mechanistic link directly. However, results of other studies support the contention that labile C amendments stimulate soil respiration and that increased respiration can be associated with increases in microbial N pools. Bowman et al. (2004) found that litter treatments with different C qualities produced a significant, positive correlation between soil respiration and microbial biomass N. Phenolic-rich LMW fractions from *Populus balsamifera* are also labile C sources for soil microbes, stimulating respiration and the immobilization of mineral N (Schimel et al. 1996, 1998; Fierer et al. 2001). Moreover, additions of labile sugars to soil can lead to increases in the microbial biomass N pool and reductions in the soil mineral N pool (Gallardo and Schlesinger 1995; Jonasson et al. 1996), although the effects of C amendments on microbial uptake of mineral N may be dependent on the levels of available mineral N in the soil (Allen and Schlesinger 2004; Dunn et al. 2006).

Phenolic concentrations across a productivity gradient

We predicted that if *A. rossii* phenolic compounds inhibited the growth of a potential competitor, then concentrations of those phenolics most inhibitory to its competitors' growth would increase as above-ground standing crop (i.e., competition intensity) increased. Two noteworthy results emerged from our productivity gradient dataset. First, total phenolic concentrations did not change with variations in the standing crop, but there were significant changes in phenolic concentrations within fractions. Similar to the results reported by Nurmi et al. (1996), our data show that potentially important trends can be missed if only total phenolics are examined.

Second, there were significant increases in phenolic concentrations within the LMW WS fractions as standing crop increased, with the largest LMW WS phenolic pools occurring in moist-meadow sites where *A. rossii* is a co-dominant, competitive equal with *D. caespitosa*. Although moist meadows are relatively resource rich, N availability limits production (Bowman et al. 1995). Within moist meadows, plant available N is lower and microbial biomass N is higher under *A. rossii* canopies than under *D. caespitosa* canopies, and *D. caespitosa* experiences a competitive release (as evidenced by a significant increase in relative abundance) when *A. rossii* is removed (Suding et al. 2008). Moreover, adding N to intact communities caused an increased relative abundance of *D. caespitosa* at the expense of *A. rossii* (Suding et al. 2008). It is therefore possible that the production of larger pools of LMW WS phenolics that inhibit N uptake could help *A. rossii* remain competitive with neighbors that have higher growth and N uptake rates (Suding et al. 2004). Although our data are consistent with this interpretation, the correlation we observed in the field does not imply causality.

Nevertheless, the potential for plants to alter the soil environment to benefit their persistence has been suggested by multiple researchers (Chapin 1980; Northup et al. 1998; Van Breemen and Finzi 1998). Because phenolic-rich LMW fractions from *A. rossii* directly inhibit neighbor root growth (and thus root N uptake) and likely reduce plant available N by influencing soil C and N cycling, there could be important feedbacks between plant phenolic production, competition with neighbors, and soil nutrient supply (Northup et al. 1998).

Conclusion

Total soluble C extracts from fresh *A. rossii* leaves and leaf litter, which were comprised mainly of sugars, LMW phenolics, and tannins, significantly increased soil respiration and also reduced the shoot growth, root growth, and leaf N concentration of *D. caespitosa* plants grown in native soil. Fractionation of the TSC extracts revealed that LMW C fractions and tannin fractions differentially influenced soil respiration and plant growth and that the LMW fractions had greater effects than tannins. An analysis of *A. rossii* leaves from populations growing across a natural productivity gradient showed that concentrations of some LMW phenolics were positively correlated with competition intensity. In addition, the effects of the fractions on soil respiration and plant growth were not additive—that is, the effects of TSC fractions were not a sum of the effects of the individual fractions.

Our results suggest two simultaneously operating mechanisms behind the effects of *A. rossii* fractions on plant growth: (1) phenolics inhibit root growth and function, thereby leading to reductions in neighbor N uptake and shoot growth; (2) labile LMW compounds (i.e., sugars, phenolics, and unidentified compounds) stimulate microbial activity and subsequently reduce plant available N. These results contribute important information to the growing body of evidence, indicating that the quality of C moving from plants to soils is a critical component of plant-mediated effects on soil biogeochemistry and, possibly, competitive interactions among species (Suding et al. 2004).

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