



# Inference of allelopathy is complicated by effects of activated carbon on plant growth

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## Summary

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Received: 3 September 2007

Accepted: 27 November 2007

- Allelopathy can play an important role in structuring plant communities, but allelopathic effects are often difficult to detect because many methods used to test for allelopathy can be confounded by experimental artifacts. The use of activated carbon, a technique for neutralizing allelopathic compounds, is now employed in tests for allelopathy; however, this technique also could produce large experimental artifacts.
- In three independent experiments, it was shown that adding activated carbon to potting media affected nutrient availability and plant growth. For most species tested, activated carbon increased plant biomass, even in the absence of the potentially allelopathic agent.
- The increased growth corresponded to increased plant nitrogen content, likely resulting from greater nitrogen availability. Activated carbon also affected nitrogen and other nutrient concentrations in soil media in the absence of plants.
- The observed effects of activated carbon on plant growth can confound its use to test for allelopathy. The detection of allelopathy relies on the difference between plant growth in medium with carbon and that in medium without carbon in the presence of the potentially allelopathic competitor; however, this difference may be biased if activated carbon alters soil nutrient availability and plant growth even in the absence of the focal allelopathic agent.

**Key words:** activated charcoal, allelopathy, biological invasion, *Centaurea solstitialis*, *Centaurea stoebe micranthos*, *Lepidium draba*.

*New Phytologist* (2008) **178**: 412–423

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doi: 10.1111/j.1469-8137.2007.02360.x

## Introduction

Several studies have demonstrated that allelopathy can play an important role in limiting plant distributions (Mahall & Callaway, 1992; Nilsson & Zackrisson, 1992), and others have argued that it promotes the success of invasive plant species in novel environments (Callaway & Aschehoug, 2000; Bais *et al.*, 2003; Hierro & Callaway, 2003). Allelopathy most commonly refers to chemically mediated interference competition between co-occurring plant species and includes

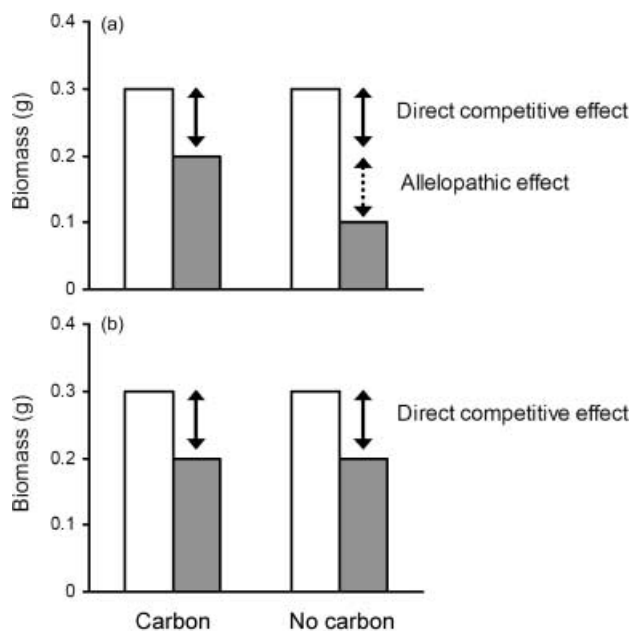
both direct effects of the chemicals and indirect effects of the chemicals that are mediated by the soil microbial community or other biota (Inderjit & del Moral, 1997). Because all plant species produce a variety of organic compounds that may be released into the environment as exudates, leachates, or volatiles, allelopathy may be a widespread phenomenon.

Although allelopathy is an appealing explanation for several negative interactions between co-occurring plants, difficulties in differentiating between allelopathy and resource competition, or other processes that can influence plant–plant interactions,

have minimized the broad acceptance of this phenomenon (Hiero & Callaway, 2003; Inderjit & Callaway, 2003). In particular, bioassays used to investigate allelopathy in the laboratory are sometimes confounded by artifacts of the methodology and may be limited in applicability to natural field conditions (reviewed in Inderjit & Callaway, 2003; Inderjit & Nilsen, 2003). Recently, an older technique for minimizing the effects of chemical exudates has been revived (Schreiner & Reed, 1907); a variety of studies have used activated carbon (AC) to neutralize the effects of allelochemicals (e.g. Mahall & Callaway, 1992; Nilsson, 1994; Callaway & Aschehoug, 2000; Inderjit & Callaway, 2003; Kulmatiski & Beard, 2006).

AC, with its large surface area and pore volume, as well as its polarity, has tremendous adsorptive capacity and complex chemical and physical properties. Its activity can be separated into adsorption, mechanical filtration, ion exchange, and surface oxidation (Cheremisinoff & Morresi, 1978). AC can be a good tool for studies of allelopathy because it acts as an adsorbent for many large organic compounds (Cheremisinoff & Morresi, 1978). Therefore, in theory, one can use AC to minimize allelopathic effects, with minor impacts on nutrient dynamics, allowing tests of allelopathy while controlling for the effects of resource competition (Inderjit & Callaway, 2003; Inderjit & Nilsen, 2003). The use of AC in allelopathy studies is especially appealing because AC can be mixed easily into soil media (e.g. Callaway and Aschehoug, 2000; Al Hamdi *et al.*, 2001; Prati & Bossdorf, 2004) or even the natural environment (e.g. Nilsson & Zackrisson, 1992; Nilsson, 1994); these procedures allow one to test for the presence of allelopathy under much more natural conditions than in many other traditional bioassays.

To determine whether a species is allelopathic using AC, one grows test plants in the presence or absence of the potentially allelopathic focal competitor crossed with the presence or absence of AC. An interaction between the competitor and AC treatments provides evidence for allelopathy when AC minimizes the effect of the competitor on test species (Fig. 1, Mahall & Callaway, 1992; Inderjit & Callaway, 2003). If the focal species is allelopathic, one would expect test species biomass to be lower in the no AC treatment than in the AC treatment when the focal competitor is present. Ideally, the AC technique provides a test for whether or not allelopathic effects occur and decrease plant growth beyond the decrease explained by resource competition. Unfortunately, if resource competition is affected by AC (as might be expected if the effects of AC on plant growth differ across species), then the magnitude of the resource competition effect (solid arrow in Fig. 1) will not remain constant across AC treatments. As a result, the assumed allelopathic effect (i.e. the difference in the magnitude of the effect of the allelopathic competitor on test species growth in the presence vs absence of AC or the difference in biomass between AC and no AC treatments when the focal competitor is present) could be confounded with the effects of AC on resource competition (Inderjit & Callaway, 2003; Inderjit & Nilsen, 2003).



**Fig. 1** Expected biomass of test species in the presence or absence of activated carbon and in the presence (closed bars) or absence (open bars) of competitors if the competitor is allelopathic (a) and if the competitor is not allelopathic (b). In the absence of activated carbon, both resource-based competition (solid arrow) and allelopathic effects (dashed arrow) decrease the growth of the focal species. In the presence of activated carbon, only resource-based competition occurs because activated carbon binds to most organic compounds, thereby minimizing any allelopathic effects. Note that this example assumes that activated carbon does not directly affect plant growth and that the magnitude of the resource competition effect remains constant across the activated carbon treatments.

Another AC-based technique to test for allelopathy is to expose test plants to allelopathic materials (chemicals, leaf extracts, or litter) in the presence or absence of resource competition crossed with the presence or absence of AC (see Nilsson, 1994). The strength of this design is that it allows estimation of the magnitude of both resource competition and allelopathic effects on plant growth; however, this approach can only be used when the specific allelochemical or allelopathic substrate can be isolated from resource competition. Also, this approach does not account for the confounding effects of AC. Given that many studies using AC in tests for allelopathy either do not present results of tests for effects of AC on plant growth in the absence of competitors or proceed to draw conclusions about allelopathy even when effects of AC on plant growth are detected, the confounding effects of AC on growth and the potential for these effects to bias tests for allelopathy deserve more investigation.

This paper combines findings from three different laboratory groups that were independently exploring the role of allelopathy in plant invasions using *Centaurea solstitialis* (yellow star

thistle), *Lepidium draba* (hoary cress), and *Centaurea stoebe micranthos* (spotted knapweed, also known as *Centaurea maculosa*). All focal species have been hypothesized to be allelopathic or are closely related to species that have been reported to be allelopathic (e.g. *Centaurea diffusa*; Callaway & Aschehoug 2000; Vivanco *et al.*, 2004). *Centaurea solstitialis* is now not thought to be allelopathic (Qin *et al.*, 2007); there is some evidence for allelopathy in *L. draba* (Qasem, 2004), and there is mixed evidence for allelopathy in *C. stoebe micranthos* (Ridenour & Callaway, 2001; Bais *et al.*, 2003, but see Blair *et al.*, 2005, 2006). Each laboratory independently conducted experiments in which test plants and focal plants (the potentially allelopathic agents) were grown with and without AC to test the effects of AC on plant growth in the absence of competitors. The specific methods employed by the laboratories differed in a number of ways, including the plant species used, the source of AC, and whether straight potting mix or potting mix inoculated with natural topsoil was used. Despite methodological differences, the results from all three laboratories were consistent in indicating that AC can affect the growth of some species even in the absence of a potentially allelopathic competitor. Importantly, these effects were observed both in simplified soil environments (potting media) and in soils inoculated with a natural soil, illustrating that AC impacts plant growth both in the presence and in the absence of natural microbial communities. To show how these growth effects can confound interpretation of studies of allelopathy, data are presented from test plants grown with and without AC and with and without the focal species *C. stoebe micranthos* in a factorial design.

In addition to reporting results from the main experiments performed in each laboratory, we also quantified the nutrient impurities present in two sources of AC, and tested the effects of AC on soil properties, the effects of AC on plant nutrient uptake, and whether impurities in the AC could account for differences in plant nutrient uptake and growth.

## Materials and Methods

### Activated carbon sources and effects of activated carbon on soil properties

Each laboratory used a different type of AC. Two types of AC had been used previously in the literature to investigate allelopathy (grade SA-30 from Carbochem Inc., Ardmore, PA, USA; Perry *et al.*, 2005; C272-212 from Fisher Scientific, Pittsburgh, PA, USA; Garnett *et al.*, 2004). The third was a commercially available water purifier grade material from Culligan International (Northbrook, IL, USA) comparable to Calgon AC (Calgon Carbon Corporation, Pittsburgh, PA, USA) used by Siemens & Blossey (2007). The total and available nitrogen (N) and phosphorous (P) in two of the AC sources (from Carbochem and Fisher Scientific) were analyzed

to measure the amounts of nutrients supplied by adding the AC to the potting media. Total N was measured on an elemental analyzer, CHN-1000 (LECO Corp., St. Joseph, MI, USA). Available N was measured by extracting a 5-g subsample with 2 N KCl and analyzing the extract for ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) on an Alpkem flow solution IV (OI Analytical, College Station, TX, USA). Total P was measured through digestion and inductively coupled plasma (ICP) analysis, and available P was measured with the Mehlich 3 method (Mehlich, 1984). Both N and P were measured in pure AC, rather than in potting media amended with AC.

More detailed studies were performed to investigate how adding AC to potting media influences several soil properties using the Carbochem AC. To determine how AC affects the nutrient availability and pH of University of California Research Mix (UC Mix = 1 part ground Canadian sphagnum, 1 part uniform coarse sand, 1 part white pumice, and 1 part redwood compost with micronutrient additions of 1.78 kg  $\text{m}^{-3}$  oyster shell, 1.78 kg  $\text{m}^{-3}$  dolomite, and 1.78 kg  $\text{m}^{-3}$  single super phosphate), we compared leachates from deionized water or deionized water amended with fertilizer flushed through pots (200-ml Ray Leach Conetainers<sup>TM</sup>; Stuewe & Sons Inc., Corvallis, OR, USA) filled with UC Mix that was or was not amended with AC. We included a fertilizer treatment because Inderjit & Callaway (2003) suggest that adding fertilizer could be useful for offsetting nutrient addition effects contributed by AC itself. Following protocols in Callaway & Aschehoug (2000), 20 ml of AC (grade SA-30 steam-activated wood; Carbochem) per 1 l of soil was added to the UC Mix by hand, resulting in approx. 1.1 g of AC per 200-ml pot in the AC treatment. When AC is added to the growing media the concentration of soil and all its components is proportionally less; however, given the small quantity of AC added, the reduced amount of growing medium should have only minor impacts on nutrient content and plant growth. There were eight replicates per treatment, yielding a total of 32 leachate samples.

Pots were initially watered to saturation with 50 ml of either deionized or fertilizer water. Two days later an additional 30 ml was added. One week later the samples were flushed with 300 ml of deionized water, and the resulting leachates were filtered and collected. The pH of each leachate was determined with a pH meter (Fisher Scientific Accumet pH meter). The leachates were then analyzed for concentrations of ammonium, nitrate, P, potassium, calcium, magnesium, sodium, zinc, copper, manganese, and iron.

Tests for the effects of Carbochem AC, fertilizer, and AC  $\times$  fertilizer interactions on each nutrient concentration and pH were conducted with ANOVA (PROC GLM; SAS Institute, Cary, NC, USA). Separate analyses were run for each nutrient and for pH. All comparisons between treatments within tests were corrected for multiple comparisons with a Tukey correction and across tests with a Bonferroni correction. Nitrate and potassium required log-transformations to meet assumptions of normality.

### Expt 1. Effects of activated carbon on the growth of test species and *Centaurea solstitialis*

To investigate potential effects of AC on plant growth in the absence of competition, we grew individual test plants in pots containing either UC Mix or UC Mix supplemented with AC. While allelopathic effects may be mediated by the microbial communities found in natural soils (reviewed in Inderjit, 2005) and while AC may alter microbial community composition (Pietikäinen *et al.*, 2000), using straight potting soil provides a simplified environment to test the effects of AC on plant growth. Following protocols in Callaway & Aschehoug (2000), 20 ml of AC (grade SA-30 steam-activated wood; Carbochem) per 1 l of soil was combined with UC Mix by hand, resulting in approx. 1.1 g of AC per 200-ml pot (conetainer™) in the AC treatment.

The test species were *Festuca idahoensis* (Elmer), *Nassella lepida* (Hitche.), *Elymus glaucus* (Buckley), *Vulpia microstachys* (Nutt.), and *Grindelia camporum* (Greene). All test species seeds were purchased from Hedgerow Farms (Winters, CA, USA); seed source populations used by this company came from local provenances in Yolo and adjoining Solano Counties, California. *Centaurea solstitialis* (L.) seeds were collected from Yolo and Santa Clara Counties, California. Twenty-five replicates per treatment for each of the test species and for *C. solstitialis* were planted, yielding an initial total of 300 experimental pots. Because some individuals failed to germinate, final sample sizes ranged from 20 to 25 per species per treatment. All pots were placed in fully randomized positions in a lathe house (a semi-protected outside growing environment).

We planted four seeds of the appropriate species into each pot and thinned to one individual per pot shortly after germination. Pots were initially misted with fertilizer solution to increase germination and survival. At the seedling stage, we switched to bottom-watering the plants with deionized water. All plants were harvested *c.* 2.5 months after planting, and the aboveground biomass of each plant was dried to constant mass at 60°C and weighed. We tested for the effects of AC on *C. solstitialis* and test-plant growth with ANOVA that included the log-transformed aboveground biomass of each species as response variables (PROC GLM; SAS Institute). AC treatment and test species were included as fixed factors, and glasshouse tray (block) was included as a random blocking factor. Because of significant test species × AC interactions, separate tests were conducted for each native test species and for *C. solstitialis*. We used a Bonferroni correction to account for multiple comparisons.

### Expts 2a and b. Effects of activated carbon on the growth of test grasses and *Lepidium draba*

These experiments were similar to Expt 1 but with different species and some differences in the methods, as outlined below. We grew individual test plants in 3-l pots containing a potting soil mix inoculated with natural topsoil (2 parts Cornell

mix (1 part peat : 2 parts vermiculite, plus sand, superphosphate, calcium flour, chelated iron, and trace elements) : 1 part topsoil : 1 part sand : 1 part perlite) with and without AC. Thus, in this experiment, a more natural microbial community is present. We added 21.7 ml of AC per 1 l of soil (Culligan International) to the potting mix using a Hobart M-800 mixer (Hobart, Troy, OH, USA), resulting in 10 g of AC per 1 l of soil. Pots were fertilized with a total of 5.2 g of Osmocote® slow-release fertilizer per 1 l of soil (14 : 14 : 14 NPK, 4-month release; Scotts Miracle-Gro, Marysville, OH, USA). The test species included 10 North American, Eurasian and Holarctic grass species in the genera *Festuca* (*Festuca arundinacea* (Schreb.), *Festuca campestris* (Rydb.), *Festuca idahoensis*, *Festuca ovina* (L.) and *Festuca rubra* (L.)) and *Poa* (*Poa annua* (L.), *Poa compressa* (L.), *Poa fendleriana* (Steudel), *Poa pratensis* (L.) and *Poa secunda* (J. Presl)). Seeds were obtained from Wind River Seed (Manderson, WY, USA) or Semences UFA Samen (Winterthur, Switzerland). We obtained *P. pratensis* seeds from both sources. *Lepidium draba* (L.) seeds were collected in 2004 from a local infestation in Latah County, ID, USA. Seeds of all species were germinated in flats filled with Cornell mix and allowed to grow for 1 wk until the formation of the first true leaves. Two seedlings were transplanted into each pot, and 1 wk later plants were thinned to one plant per pot.

Experiments were conducted separately for the five *Festuca* species and *L. draba* (Expt 2a) and the five *Poa* species and *L. draba* (Expt 2b). Six replicates for each of the *Festuca* species and 18 replicates of *L. draba* per treatment (a total of 96 pots) were transplanted in August 2005 and arranged in a completely randomized block design. Six replicates of each *Poa* species and 36 replicates of *L. draba* per treatment (a total of 144 pots) were transplanted and arranged in a completely randomized block design in September 2005. Both sets of plants were kept in a climate-controlled glasshouse with a 15 : 9 h light:dark photoperiod at 24 ± 2°C (day) : 18 ± 1°C (night). All plants were harvested 85 ± 1 d after transplanting. The shoot biomass of each plant was weighed to the nearest 0.01 g after drying for a minimum of 24 h at 80°C. Statistical analyses were the same as for Expt 1.

### Expt 3. Effects of activated carbon on growth of test grasses and *C. stoebe micranthos*, effects of AC on plant nutrient content, and a test for allelopathic interactions

The goals of this experiment were: (1) to test for effects of AC on plant growth, (2) to examine how AC influences nutrient availability, and (3) to investigate how the impacts of AC on both test and focal species growth might alter resource competition and therefore change the outcome of competitive interactions and the interpretation of results of allelopathy studies. This experiment was similar to the first two, except that in addition to growing the test plants with and without AC, we also included a competition treatment using *Centaurea stoebe*

(L.) ssp. *micranthos* in a fully crossed design. Thus, there were four treatments: no competitor, no AC; no competitor, AC added; *C. stoebe micranthos*, no AC; *C. stoebe micranthos*, AC added. As outlined in Fig. 1, a significant interaction between the *C. stoebe micranthos* and AC treatments, where AC minimizes the effect of *C. stoebe micranthos* on the grasses, provides evidence that *C. stoebe micranthos* is allelopathic. In particular, if a focal species is allelopathic, the test species is expected to grow more when AC is added compared with no AC treatments when the focal species is present because the AC is expected to reduce allelochemical concentrations. We used six test species: *Setaria viridis* (L.), *Bromus tectorum* (L.), *Agropyron cristatum* (L.), *Agropyron smithii* (Rydb.), *Bouteloua gracilis* (Kunth), and *Andropogon gerardii* (Vitman). *Agropyron smithii* seeds were collected from Oklahoma, *A. gerardii* seeds were collected from Kansas, and seeds of the other grasses were purchased from Pawnee Butte Seed, Inc. (Greeley, CO, USA). *Centaurea stoebe micranthos* seeds were collected from California.

Plants were grown in 30 × 7.5 cm pots (conetainers<sup>TM</sup>), in a potting mix of 70 parts fine silica sand and 30 parts Metro Mix 350 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA; vermiculite, Canadian sphagnum peat moss, processed bark ash, starter nutrient charge, dolomitic limestone and wetting agent). Thus, as in Expt 1, a simplified soil environment was used. The AC treatment included, by volume, 10 ml of powdered AC (C272-212; Fisher Scientific) per l of the potting mix (following Callaway & Aschehoug, 2000), or approx. 2.8 g of AC per pot. As above, AC was hand-mixed into the potting medium. Metro-mix contains some charcoal, and if this charcoal also acts to adsorb chemicals, then the non-AC treatment also may have had reduced allelochemical concentrations.

All pots were planted with 10 grass seeds each, and were thinned to one test individual per pot, as seedlings emerged. Between three and five *C. stoebe micranthos* seeds (thinned to one individual as seedlings emerged) were planted into half of the pots in each of the AC treatments. Each of the four treatments (AC presence/absence × *C. stoebe micranthos* presence/absence) was replicated 24 times per test species. Because of differences in germination, replication ranged from 17 to 29 individuals per treatment. In the glasshouse, pots were grouped by test grass species and randomized across treatments. All plants were adequately watered, but no fertilizers were used over the course of the experiment. After 3 months, the plants were harvested, and above- and belowground tissues of both focal and test species were dried and weighed.

We analyzed the effects of AC on ln-transformed grass biomass in the absence of *C. stoebe micranthos*, as done for Expts 1 and 2. Harvest date was included as a covariate because it took several days to complete the harvest. While not initially planned, because of poor germination of some grass seed, we also were able to determine how AC affected *C. stoebe micranthos* biomass. To examine potential allelopathic interactions, we ran a model that included ln-transformed grass biomass as the response variable, and AC treatment, competition treatment,

test species, and all interactions as fixed factors, with harvest date included as a covariate.

Additionally, for this experiment, we analyzed the N and P concentrations in both above- and belowground tissues for a subset of test individuals grown without competition to evaluate whether the nutrients added as impurities in the AC (see AC analysis results below) or effects of AC on nutrient availability could account for enhanced growth in the presence of AC ( $n = 2$  plants per species for N;  $n = 3$  per species for P). Because plants were grown from seed, the amount of N and P in their biomass largely represents plant nutrient uptake during the experiment, and plant nutrient uptake should be largely a function of nutrient availability in this experiment. Nitrogen concentration was measured by elemental analysis on a CHN-1000 (LECO Corp.). Phosphorus concentration was measured by inductively coupled plasma analysis of acid digestions of the tissues (Thermo Jarrell-Ash IRIS Advantage Dual View High Resolution ICP; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total biomass N and P were calculated by multiplying above- and belowground biomass by the nutrient concentrations for each tissue type, and then adding the two tissue types together. Total nutrient concentrations are more informative regarding nutrient uptake and availability than nutrient concentration within tissues, as increased growth can dilute tissue nutrient concentration. These data on total nutrient concentrations, in conjunction with the elemental analyses of the AC sources (see Results below), can indicate whether the amount of N or P added as impurities in the AC explains the effects of AC on plant nutrient uptake and growth. We note here, however, that AC can also stimulate N mineralization, which may limit our ability to determine whether any observed differences in nutrient uptake are the result of direct versus microbe-mediated indirect effects. Effects of AC on total biomass N and P were analyzed with ANOVA. AC treatment, species, and their interaction were included as fixed factors.

## Results

### Activated carbon sources and effects of activated carbon on soil properties

The Carbochem AC contained 0.637% N, and the Fisher Scientific AC contained 0.549% N. Available N from both AC sources was also low; 1.93 and 1.21  $\mu\text{g N g}^{-1}$  AC, respectively. Thus, the additions of N to pots in the AC treatments were small: 2.2  $\mu\text{g N}$  in the experiments using the Carbochem AC (the experiment testing AC effects on soil properties and Expt 1), and 3.44  $\mu\text{g N}$  in Expt 3 using the Fisher AC.

Additions of P with AC were larger: the Carbochem AC contained 371  $\mu\text{g}$  of available P per g, and the Fisher AC contained 21.5  $\mu\text{g}$  of available P per g. Thus, adding AC resulted in an extra 423  $\mu\text{g}$  of P per pot for experiments using the Carbochem AC and 60  $\mu\text{g}$  per pot for experiments using the Fisher AC.

**Table 1** Least-squares means  $\pm$  SE for pH and nutrient concentrations of leachates from soils in the two activated carbon (AC) treatments

Soil property	<i>Fertilized</i>		<i>Nonfertilized</i>		<i>Treatment effects</i>	
	Carbon	No carbon	Carbon	No carbon	Carbon	Carbon $\times$ fert.
pH	<b>6.02 <math>\pm</math> 0.02</b>	<b>5.70 <math>\pm</math> 0.02</b>	<b>5.98 <math>\pm</math> 0.02</b>	<b>5.53 <math>\pm</math> 0.02</b>	Yes	No*
Ammonium (mg l <sup>-1</sup> )	<b>3.44 <math>\pm</math> 0.09</b>	<b>4.90 <math>\pm</math> 0.09</b>	0.90 $\pm$ 0.09	1.25 $\pm$ 0.09	Yes	Yes
Log(nitrate)	3.73 $\pm$ 0.09	3.63 $\pm$ 0.09	<b>-1.25 <math>\pm</math> 0.09</b>	<b>-2.37 <math>\pm</math> 0.09</b>	Yes	Yes
Phosphorus (mg l <sup>-1</sup> )	22.16 $\pm$ 0.43	21.53 $\pm$ 0.46	20.48 $\pm$ 0.43	18.94 $\pm$ 0.43	No*	No
Log(potassium (mg l <sup>-1</sup> ))	<b>4.08 <math>\pm</math> 0.01</b>	<b>3.75 <math>\pm</math> 0.01</b>	<b>2.93 <math>\pm</math> 0.01</b>	<b>2.22 <math>\pm</math> 0.01</b>	Yes	Yes
Calcium (meq l <sup>-1</sup> )	<b>1.78 <math>\pm</math> 0.02</b>	<b>2.10 <math>\pm</math> 0.03</b>	<b>0.61 <math>\pm</math> 0.02</b>	<b>0.82 <math>\pm</math> 0.02</b>	Yes	No*
Magnesium (meq l <sup>-1</sup> )	2.10 $\pm$ 0.03	2.23 $\pm$ 0.03	<b>0.58 <math>\pm</math> 0.03</b>	<b>0.74 <math>\pm</math> 0.03</b>	Yes	No
Sodium (meq l <sup>-1</sup> )	1.62 $\pm$ 0.03	1.51 $\pm$ 0.03	1.34 $\pm$ 0.03	1.26 $\pm$ 0.03	No*	No
Iron (mg l <sup>-1</sup> )	<b>0.10 <math>\pm</math> 0.02</b>	<b>0.03 <math>\pm</math> 0.02</b>	<b>0.31 <math>\pm</math> 0.02</b>	<b>0.09 <math>\pm</math> 0.02</b>	Yes	Yes
Zinc (mg l <sup>-1</sup> )	<b>0.13 <math>\pm</math> 0.01</b>	<b>0.29 <math>\pm</math> 0.01</b>	–	–	Yes	–
Manganese (mg l <sup>-1</sup> )	<b>0.20 <math>\pm</math> 0.01</b>	<b>0.27 <math>\pm</math> 0.01</b>	–	–	Yes	–

Values shown are for both fertilized and nonfertilized samples, when available. Significant treatment effects are indicated as follows: Yes =  $P < 0.0042$  (Bonferroni adjustment for experiment-wide significance at  $P < 0.05$ ), No =  $P > 0.0042$ , No\* =  $P < 0.05$ , before Bonferroni correction.

Values in bold indicate significant pairwise differences between carbon treatments, corrected for multiple comparisons within tests with a Tukey correction and across tests with a Bonferroni correction.  $df = 1, 27$  except for the response variable pH, where  $df = 1, 8$ .

The Carbochem AC significantly increased potting medium pH (Table 1). In addition, AC increased concentrations of nitrate, potassium, and iron, but decreased concentrations of ammonium, calcium, magnesium, zinc, and manganese (Table 1). In some cases, there were significant interactions between the AC and fertilizer treatments, such that the magnitude of the AC effect depended on fertilizer treatment. For example, the effect of AC on nitrate concentrations was larger in magnitude (67% increase vs a 10% increase) and only statistically significant in nonfertilized conditions. By contrast, the effect of AC on ammonium was slightly greater (42% decrease vs 39% decrease) in the fertilized treatment. The addition of P as an impurity in the AC did not translate into significant increases in P concentrations of leachates (Table 1). These effects on soil medium properties were measured over a short period (1 wk). AC thus had an immediate effect on nutrient availability and also could have an effect over a longer period of time.

### Effects of activated carbon on plant growth

In all three experiments, amending potting media with AC altered growth of some plants, most often by increasing biomass by more than 33% on average (Table 2a, Fig. 2). Thirteen of 26 comparisons showed growth differences at  $P < 0.05$ , with four of those remaining significant after conservative Bonferroni adjustment.

In Expt 1 there was a significant AC  $\times$  test species interaction ( $F_{4,232} = 2.46$ ,  $P = 0.046$ ), indicating that AC altered plant growth and that the magnitude and/or direction of the effect varied across test species (Fig. 2a). Adding AC to the soil

decreased the aboveground biomass of *G. camporum* by 40%, but increased the biomass of *V. microstachys* by 60%.

In Expt 2a, AC significantly increased biomass ( $F_{1,72} = 36.98$ ,  $P < 0.001$ ), but the magnitude of that increase depended upon the test species (AC  $\times$  test species interaction,  $F_{5,72} = 3.87$ ,  $P = 0.004$ ). In Expt 2b both the direction and the magnitude of effects of AC on biomass were species specific (AC  $\times$  test species interaction,  $F_{6,124} = 8.36$ ,  $P < 0.001$ ). When individual species were examined across Expt 2, all significant effects of AC were found to be associated with increases in plant growth (Table 2b,c, Fig. 2b,c). Interestingly, the growth trends were opposite for *P. pratensis* from European and North American sources (Fig. 2c).

In Expt 3, in the absence of competition, AC significantly increased aboveground biomass ( $F_{1,260} = 39.21$ ,  $P < 0.0001$ ), although the magnitude of the increase varied among test species (AC  $\times$  test species interaction,  $F_{5,260} = 2.44$ ,  $P = 0.035$ ). AC significantly increased the aboveground biomass of *B. tectorum*, *B. gracilis*, and *A. gerardii*, with *S. viridis* and *A. cristatum* also exhibiting similar, marginally significant trends (Table 2d, Fig. 2d). For all three experiments, AC also tended to increase the growth of the potentially allelopathic focal species (Fig. 2), although this growth increase was only statistically significant after Bonferroni correction for *L. draba*.

### Can impurities in activated carbon account for differences in plant growth?

In Expt 3, the total N content of the plants grown with AC was greater than for the plants grown without AC ( $7.22 \pm 0.40$  mg

**Table 2** ANOVA of the effects of activated carbon on the biomass of the test species used in all three laboratories: (a) Expt 1, (b) Expt 2a, (c) Expt 2b and (d) Expt 3

(a)											
Source	df	<i>Vulpia microstachys</i>		<i>Grindelia camporum</i>		<i>Elymus glaucus</i>		<i>Festuca idahoensis</i>		<i>Nassella lepida</i>	
		F	P	F	P	F	P	F	P	F	P
Carbon	1	<b>5.14</b>	<b>0.029</b>	<b>6.52</b>	<b>0.0148</b>	0.09	0.769	0.34	0.564	0.48	0.491
Block	6	3.61	0.006	2.28	0.056	7.52	< 0.0001	1.79	0.126	2.47	0.040

(b)											
Source	df	<i>Festuca arundinacea</i>		<i>Festuca campestris</i>		<i>Festuca idahoensis</i>		<i>Festuca ovina</i>		<i>Festuca rubra</i>	
		F	P	F	P	F	P	F	P	F	P
Carbon	1	0.55	0.493	<b>210.29</b>	<b>&lt; 0.001*</b>	6.74	0.081	0.49	0.533	<b>8.39</b>	<b>0.034</b>
Block	5	1.70	0.287	25.92	0.004	0.95	0.554	1.63	0.364	1.23	0.413

(c)													
Source	df	<i>Poa annua</i>		<i>Poa compressa</i>		<i>Poa fendleriana</i>		<i>Poa pratensis</i> (NA)		<i>Poa pratensis</i> (EU)		<i>Poa secunda</i>	
		F	P	F	P	F	P	F	P	F	P	F	P
Carbon	1	2.36	0.185	<b>13.78</b>	<b>0.014</b>	0.88	0.402	0.57	0.485	4.17	0.097	0.00	0.955
Block	5	1.47	0.342	0.50	0.765	0.18	0.958	0.36	0.855	0.48	0.780	1.12	0.451

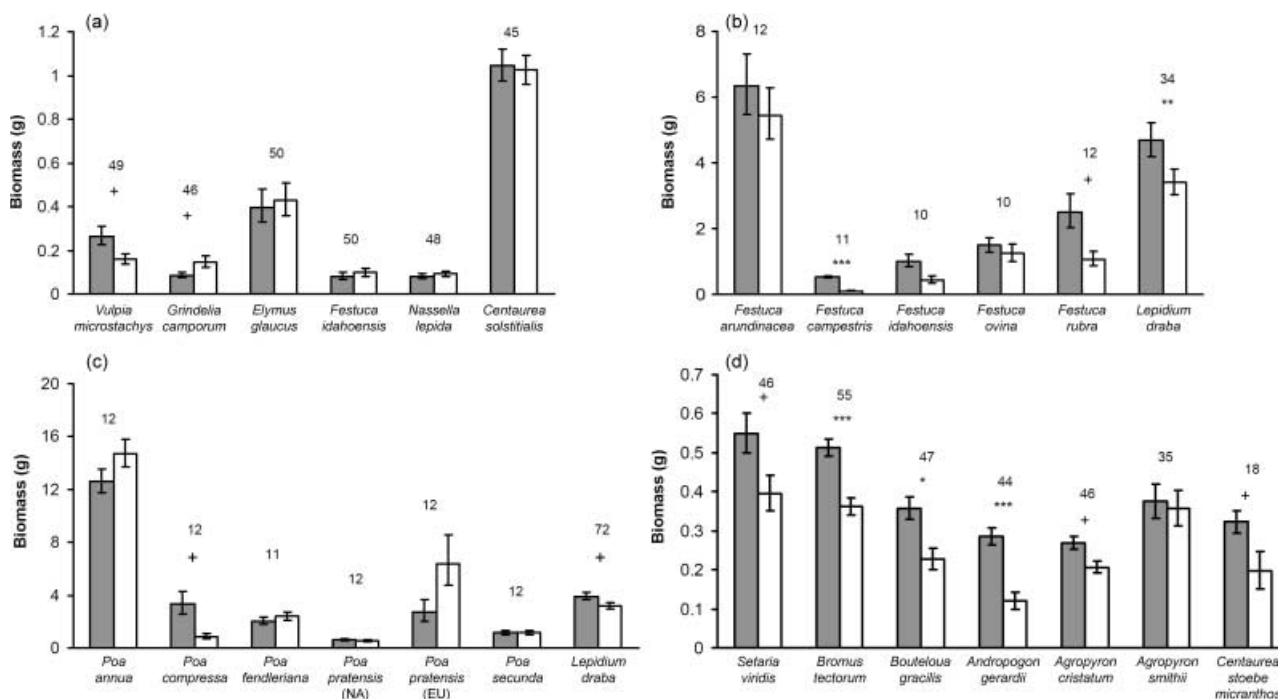
(d)													
Source	df	<i>Setaria viridis</i>		<i>Bromus tectorum</i>		<i>Bouteloua gracilis</i>		<i>Andropogon gerardii</i>		<i>Agropyron cristatum</i>		<i>Agropyron smithii</i>	
		F	P	F	P	F	P	F	P	F	P	F	P
Carbon	1	<b>5.11</b>	<b>0.030</b>	<b>22.75</b>	<b>0.0001*</b>	<b>10.86</b>	<b>0.0019*</b>	<b>28.04</b>	<b>0.0001*</b>	<b>6.86</b>	<b>0.012</b>	0.08	0.780
Harvest date	1	5.22	0.027	0.51	0.477	1.33	0.256	1.88	0.178	0.05	0.825	4.72	0.037

Significant carbon effects are shown in bold. *P*-values with an asterisk indicate effects that remain significant following Bonferroni adjustments for experiment-wide significance at  $P < 0.05$  ( $P < 0.01$  for a and b,  $P < 0.0083$  for c and d). In Expt 2b, *Poa pratensis* seeds were obtained from both North American (NA) and European (EU) sources.

versus  $5.31 \pm 0.40$  mg;  $F_{1,12} = 11.7$ ,  $P = 0.005$ ). The amount of N in the AC itself ( $3.44 \mu\text{g N}$  per pot) is an order of magnitude lower than the difference observed and cannot account for the greater total N content of the plants grown in AC-amended soils. This suggests that amending potting mix with AC likely increased the mineralization of N from organic N sources in the potting mix. By contrast, averaged across all species, AC did not significantly affect the total P content of the plants ( $880 \pm 70.9 \mu\text{g}$  versus  $774 \pm 70.9 \mu\text{g}$ ;  $F_{1,1} = 1.13$ ,  $P = 0.3$ ). We did, however, detect a significant AC  $\times$  test species interaction for total P content ( $F_{5,5} = 4.15$ ,  $P = 0.0074$ ). *Setaria viridis* and *A. smithii* tended to have reduced total P content in AC-amended soils, although these differences were not significant. By contrast, *B. tectorum*, *B. gracilis*, *A. gerardii*, and *A. cristatum* tended to have higher total P content in AC-amended soils, although this effect was only statistically significant for *A. gerardii* ( $F_{1,1} = 49.23$ ,  $P = 0.002$ ).

#### Test for allelopathic effects of *C. stoebe micranthos*

Competition with *C. stoebe micranthos* significantly decreased the aboveground biomass of the six test species used in Expt 3 (Table 3, Fig. 3). Additionally, there were significant interactions between AC and competition treatments for *B. tectorum*, *B. gracilis*, and *A. gerardii* (Table 3, Fig. 3). Test species biomass did not differ between AC treatments when *C. stoebe micranthos* was present. Thus, we detected no evidence that allelopathic effects (which can occur when AC is absent) decrease test plant growth. Test species biomass did differ between AC treatments when competitors were absent, however. As a result, the relative effect of *C. stoebe micranthos* on test plant growth was greater in the presence of AC (i.e. *C. stoebe micranthos* was more inhibitory in the presence of AC than in its absence). On the surface, this result is inconsistent with allelopathy. It is also possible, however, that positive



**Fig. 2** Least-squares means  $\pm$  SE showing effects of activated carbon on plant biomass in three separate experiments: (a) Expt 1, (b, c) Expt 2 and (d) Expt 3 (see text for details). Closed columns, carbon; open columns, no carbon. Numbers above columns are sample sizes. Plants were grown in the absence of competitors. Significant differences between carbon treatments are indicated as follows: +,  $P < 0.05$  before Bonferroni correction. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , post-Bonferroni corrections.

effects of AC on the growth of both *C. stoebe micranthos* and the test species obscured allelopathic effects. Notably, the three test species for which we detected interactions between AC additions and competition were also the same species that responded most strongly to the presence of AC alone. The increased inhibitory effect of *C. stoebe micranthos* in the presence of AC could be a result of the positive effect of AC on test species biomass which was only observed when *C. stoebe micranthos* was absent. The positive effect of AC on test species biomass might not have been observed when *C. stoebe micranthos* was present because *C. stoebe micranthos* also tended to respond positively to AC.

## Discussion

Historically, the study of allelopathy has been plagued by difficulties in separating resource competition from allelochemical interference (Inderjit & del Moral, 1997; Inderjit & Callaway, 2003). Recently, AC has been used to test for allelopathic effects on plant growth; however, use of AC to test for allelopathy may lead to erroneous results if one does not control for confounding effects of AC on both test and focal species growth.

Most studies using AC in allelopathy experiments do test for effects of AC on the growth of test species in the absence of the potentially allelopathic agent to control for unwanted

growth effects of AC that accompany its intended use as a minimizer of allelopathic effects. Roughly half (eight of 17) of the studies we examined, however, either did not present results of tests for effects of AC on growth or failed to test for direct effects on all species (both focal and test) used in the experiment. Furthermore, several studies interpreted tests for allelopathy even when AC affected plant growth in the absence of the putatively allelopathic agent.

Our results demonstrate that AC affects the growth of several plant species, likely via its effects on potting medium properties, especially nutrient availability. Inderjit & Callaway (2003) recommend fertilizing pots to minimize the effects of trace concentrations of nutrients contributed by AC. However, our results show that AC significantly influenced some nutrient concentrations in both the presence and the absence of fertilizer (Table 1). Furthermore, the results from N and P analyses of two sources of AC (for Expts 1 and 3) and for plant tissues from Expt 3 suggest that direct addition of nutrients along with the AC is likely to be relatively minor, at least for the two sources of AC that we had analyzed. Other studies have documented that charcoal or AC can increase nitrification, in part because charcoal may sorb compounds that are inhibitory to nitrifying bacteria (DeLuca *et al.*, 2002, 2006; Berglund *et al.*, 2004; Gundale & DeLuca, 2007). Notably, these effects depend on the availability of other nutrients (N sources), and effects on plant growth even varied across substrates and

**Table 3** ANOVA of the effects of activated carbon, the presence or absence of *Centaurea stoebe micranthos*, and their interaction on the biomass of the six test species used in Expt 3

Source	df	Setaria viridis		Bromus tectorum		Bouteloua gracilis		Andropogon gerardii		Agropyron cristatum		Agropyron smithii	
		F	P	F	P	F	P	F	P	F	P	F	P
<i>Centaurea stoebe micranthos</i>	1	<b>199.42</b>	<b>&lt; 0.0001</b>	<b>394.12</b>	<b>&lt; 0.0001</b>	<b>204.59</b>	<b>&lt; 0.0001</b>	<b>160.89</b>	<b>&lt; 0.0001</b>	<b>236.14</b>	<b>&lt; 0.0001</b>	<b>156.5</b>	<b>&lt; 0.0001</b>
Carbon	1	6.48	0.0126	<b>27.29</b>	<b>&lt; 0.0001</b>	<b>11.64</b>	<b>0.0010</b>	<b>27.65</b>	<b>&lt; 0.0001</b>	3.71	0.0576	0.05	0.8319
Carbon × <i>Centaurea stoebe micranthos</i>	1	3.91	0.051	<b>9.36</b>	<b>0.0028</b>	<b>8.14</b>	<b>0.0054</b>	<b>30.84</b>	<b>&lt; 0.0001</b>	5.76	0.0186	0.03	0.8623
Harvest date	1	6.25	0.0142	0.42	0.5173	1.20	0.2768	2.17	0.1448	0.02	0.8786	4.93	0.0297

Significant carbon and *Centaurea stoebe micranthos* effects are shown in bold ( $P < 0.0083$ , Bonferroni adjustment for experiment-wide significance at  $P < 0.05$ ).

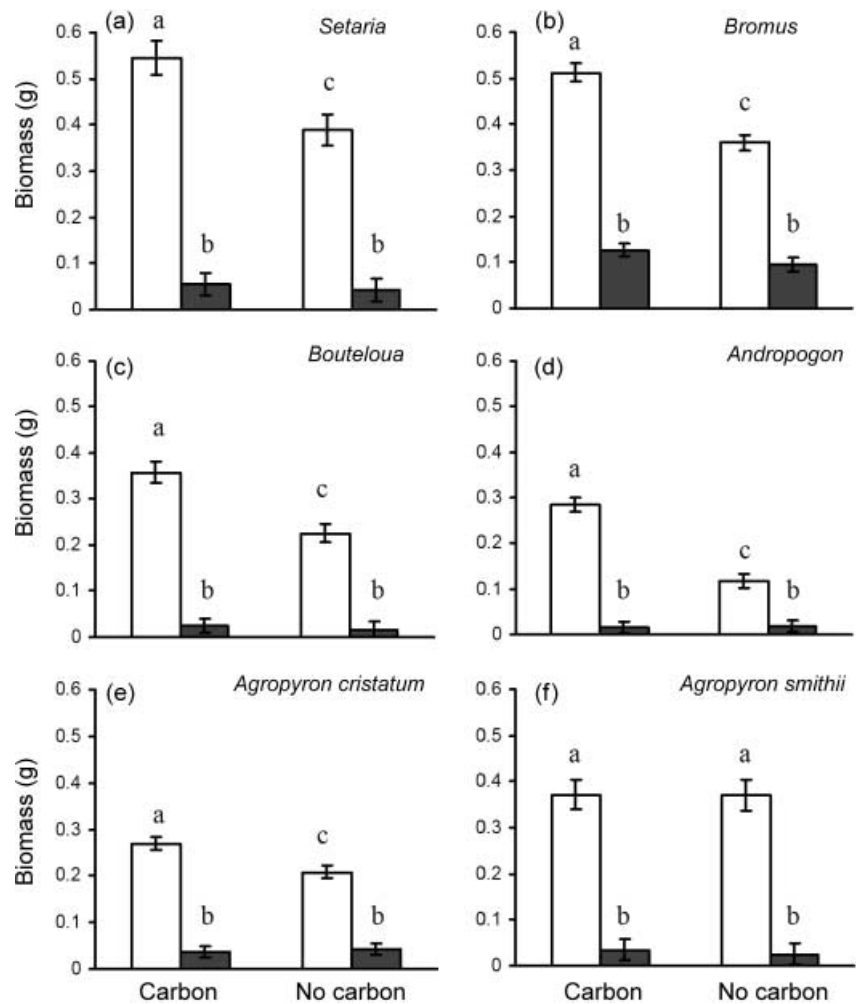
depended on the source material of the charcoal (Wardle *et al.*, 1998; Gundale & Deluca, 2007).

There are other mechanisms besides altered nutrient availability that could contribute to the effects of AC on plant growth. For example, the potting media used in these experiments contained organic matter (sphagnum and redwood compost) that may have allelopathic properties. Therefore, the positive effect of AC on growth could be a result of AC minimizing any negative growth effects of these compounds. Another possible mechanism underlying our findings is a change in microbial community composition in the presence of AC (e.g. Pietikäinen *et al.*, 2000) which might affect plant growth.

Whatever the underlying mechanism(s), we found that AC influenced plant growth in the absence of the putative allelopathic species over the widely varying experimental conditions used in the three laboratories (e.g. with and without additions of fertilizer, with and without inoculation of potting mix with natural field soils). These effects of AC on plant growth could bias tests for allelopathy, leading to erroneous conclusions that either suggest allelopathy is acting when it is not, or prevent the detection of allelopathy when it is truly present. The type of bias may depend largely upon whether the test species and the potentially allelopathic species demonstrate positive or negative responses to AC.

If a test species responds positively to AC, relatively increased growth in the presence of AC and a potentially allelopathic competitor could be interpreted wrongly as evidence for allelopathy. The patterns may not always be that simple, however. Positive responses to AC may also obscure allelopathic effects, as the increased growth in the absence of a competitor means that the proportional reduction in biomass in the presence of the competitor can be greater with AC than without AC. For example, for all test species that responded positively to AC in Expt 3, we detected an interaction between *C. stoebe micranthos* and AC on test species growth, such that the effects of *C. stoebe micranthos* were heightened in the presence of AC (Fig. 3b,c,d), rather than reduced as would be expected if *C. stoebe micranthos* were allelopathic. In a related study using similar methods, the same pattern was observed with *V. microstachys*, which responded positively to AC in Expt 1 (Qin *et al.*, 2007).

By contrast, when test species respond negatively to AC, interactions between AC and competitors may erroneously suggest either allelopathy or the absence of allelopathy, depending on whether one focuses on the effects of AC on test growth in the presence of the focal species or on the magnitude of the reduction of test species growth resulting from the presence of the focal species in AC compared to no AC treatments. For example, *G. camporum* responded negatively to AC in Expt 1, and the effect of competition from *C. solstitialis* on *G. camporum* biomass tended to be greater in the absence of AC than in the presence of AC (Qin *et al.*, 2007), as would be expected were *C. solstitialis* allelopathic. This outcome was



**Fig. 3** Least-squares means  $\pm$  SE showing the effects of activated carbon and *Centaurea stoebe micranthos* on the growth of six test species: (a) *Setaria viridis*, (b) *Bromus tectorum*, (c) *Bouteloua gracilis*, (d) *Andropogon gerardii*, (e) *Agropyron cristatum* and (f) *Agropyron smithii*. Open columns, *C. stoebe micranthos* absent; closed columns, *C. stoebe micranthos* present. Significant interactions between activated carbon and *C. stoebe micranthos* occurred for *B. tectorum*, *B. gracilis* and *A. gerardii* (Table 3). Bars with similar letters indicate that treatments do not differ significantly at  $P < 0.05$ , adjusted for multiple comparisons within tests with a Tukey correction.

likely driven as much by the negative main effects of AC on *G. camporum* growth as by any possible allelopathic effects of *C. solstitialis*, especially given that tests for allelopathy in *C. solstitialis* employing alternative methods also failed to detect strong allelopathic effects on a multitude of test species (Qin *et al.*, 2007).

If a potentially allelopathic species responds positively to AC, then resource competition could be strengthened in the AC treatment, which would mask allelopathy. In essence, because the magnitude of the resource competition effect does not remain constant across AC treatments (in contrast to what is depicted in Fig. 1), it becomes impossible to identify whether a portion of the competitive effect is a result of allelopathy. This may play a role in our results from Expt 3, as *C. stoebe micranthos* responded favorably to AC. We failed to detect allelopathic effects of *C. stoebe micranthos* on any test species, despite the fact that others have suggested that the plant is strongly allelopathic (Ridenour & Callaway, 2001; Hierro & Callaway, 2003, but see Blair *et al.*, 2005, 2006), including

activity against one of the test species used here (*B. tectorum*; Bais *et al.*, 2002). Certainly, methodological differences contribute to the disparity between our results and those of the others. These conflicting results, however, highlight that methodological details and effects of AC on growth are important considerations in experiments investigating allelopathy.

The test is also biased if a potentially allelopathic species responds negatively to AC. In this case, however, the focal species may have a reduced competitive effect on the test species in the presence of AC, a situation that would mimic allelopathy, but may, in fact, be a result of biomass effects and resource competition. Depending on the limiting nutrients for the focal and test species, AC (and its associated effects) could shift the competitive balance toward either species.

To complicate matters further, the effects of AC on a species may not be consistent from experiment to experiment. In this study, AC significantly increased the growth of *C. stoebe micranthos*. Interestingly, negative effects of AC on *C. stoebe micranthos* have also been documented (Newingham &

Callaway, 2006). Also, in this study, AC was found to significantly enhance growth of *L. draba* in Expt 2a, but to have only a modest positive effect that was not significant after Bonferroni correction in Expt 2b. Thus, the growth effects of AC can be context dependent and can vary across differing experimental conditions.

In summary, our results suggest that conclusions drawn about allelopathy based on the use of AC may be suspect if AC impacts the growth of either the test or hypothesized allelopathic focal species when grown in the absence of competition. Given the inherent problems of AC and many of the other techniques used in investigations of allelopathy, studies using a multitude of techniques with different potential artifacts and confounding factors (e.g. gel filtration, watering with plant extracts, root chamber experiments, and AC) under different environmental conditions provide the most compelling evidence for (or against) allelopathy, if they agree (e.g. Nilsson & Zackrisson, 1992; Qin *et al.*, 2007). Furthermore, experiments in which the hypothesized allelochemical is isolated and can be manipulated in the field provide powerful tests of the existence and relevance of allelopathy under field conditions. One of the few cases in which the chemical has been isolated (e.g. Bais *et al.*, 2002, 2003; Vivanco *et al.*, 2004) is controversial, however, as the results are not consistent across different laboratories (Blair *et al.*, 2005, 2006) and field concentrations of the proposed allelochemical vary widely and are often extremely low or undetectable (Perry *et al.*, 2007). Because of the large and multifarious effects of AC on potting medium nutrients and plant growth, conclusions drawn about allelopathy are most convincing when AC is used in conjunction with other experimental techniques and with some understanding of the mechanism underlying allelopathic effects.

## Acknowledgements

We thank Lisa Passerello for glasshouse and logistic assistance, Cindy Roessler of the MROSD for logistical support with seed collections, and Ray Callaway and three anonymous reviewers for providing comments on previous drafts of this manuscript. This work was supported by the UC Davis Biological Invasions IGERT NSF-DGE 0114432 and the UC Davis President Undergraduate Fellowship (PUF Grant), and Idaho State Department of Agriculture through its cost share program and USDA NRI grant IDA00108-CG to MS, and by Colorado Agriculture Experiment Station grant COL00180 and USDA NRI grant 2002-00672 to RAH. This is Kellogg Biological Station contribution #1449.

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