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Roots of the Invasive Species *Carduus nutans* L. and *C. acanthoides* L. Produce Large Amounts of Aplotaxene, a Possible Allelochemical

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Abstract The invasive thistle *Carduus nutans* has been reported to be allelopathic, yet no allelochemicals have been identified from the species. In a search for allelochemicals from *C. nutans* and the closely related invasive species *C. acanthoides*, bioassay-guided fractionation of roots and leaves of each species were conducted. Only dichloromethane extracts of the roots of both species contained a phytotoxin (aplotaxene, (Z,Z,Z)-heptadeca-1,8,11,14-tetraene) with sufficient total activity to potentially act as an allelochemical. Aplotaxene made up 0.44 % of the weight of greenhouse-grown *C. acanthoides* roots (*ca*. 20 mM in the plant) and was not found in leaves of either species. It inhibited growth of lettuce 50 % (I_{50}) in soil at a concentration of ca. 0.5 mg g^{-1} of dry soil (*ca*. 6.5 mM in soil moisture). These values gave a total activity in soil value (molar concentration in the plant divided by the molarity required for 50 % growth inhibition in soil = 3.08) similar to those of some established allelochemicals. The aplotaxene I_{50} for duckweed (*Lemna paucicostata*) in nutrient solution was less than 0.333 mM, and the compound caused cellular leakage of cucumber cotyledon discs in darkness and light at similar concentrations. Soil in which *C. acanthoides* had grown contained aplotaxene at a lower concentration than necessary for biological activity in our short-term soil bioassays, but these levels might have activity over longer periods of time and might be an underestimate of concentrations in undisturbed and/or rhizosphere soil.

Keywords Allelochemical · Allelopathy · Aplotaxene · *Carduus nutans* · *Carduus acanthoides* · Phytotoxin

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Introduction

Carduus nutans L. (musk thistle or nodding thistle), a member of the Asteraceae, is a Eurasian native plant (Desrochers et al. 1988) that is an invasive species in North America, Australia, and New Zealand, where it is more competitive in many habitats than in its native area (Jongejans et al. 2006, 2008; Rauschert et al. 2012; Shea et al. 2005). Its competitive ability was independent of whether it is 2,4-D resistant (Bonner et al. 1998). Part of its success may be due to allelopathy (Formisano et al. 2007; Wardle et al. 1994, 1998), even though it can be negatively affected by the phytochemicals (perhaps allelochemicals) produced by competing plant species

(Wardle et al. 1996). *Carduus acanthoides* L. (spiny plumeless thistle), another invasive *Carduus* species of Eurasian origin that causes less economic damage (Rauschert et al. 2012; Skarpaas and Shea 2007), is closely related to *C. nutans* and can genetically cross with it where it co-exists (Mulligan and Moore 1961; Warwick et al. 1989), although the two species differ in chromosome numbers, and the hybrids are mostly sterile. There are no reports of allelopathic properties of this species. Several secondary compounds have been identified in the genus *Carduus*. Among them are sterols such as taraxasterol and sitosterol, and flavonoids such as kaempferol, apigenin, and rutin (Abdallah et al. 1989; Bain and Desrochers 1988; Jorodon-Thaden and Louda 2003: Kaloshina and Mazulin 1988).

The novel weapons hypothesis proposes that one reason for the success of some invading plant species is that they produce an allelochemical to which native plants have not co-evolved defenses (Callaway and Ridenour 2004). Despite the strong interest in these *Carduus* species because they are invasive, there has been little effort to identify specific allelochemicals. All of the allelopathy studies but one have involved aqueous extracts of *C. nutans* as test material (Wardle et al. 1991, 1993). Hydrophilic compounds may be less effective allelochemicals than lipophilic compounds because they can readily leach from soil (Dayan and Duke 2009; Duke 2010). Thus, these previous studies may have missed the most important allelochemical(s). The essential oil from the shoot of *C. nutans* is mildly phytotoxic and contains known phytotoxins such as eugenol (Formisano et al. 2007), but root chemistry was not described in this paper. In the present study, bioassay-guided isolation was used to discover phytotoxic compounds from both *Carduus* species that might be involved in allelopathy. We report that the roots of both species produce relatively high amounts of the weakly phytotoxic, highly lipophilic compound aplotaxene [(Z,Z,Z)-heptadeca-1,8,11,14-tetraene] (Fig. 1). Aplotaxene was not present in the leaves of either species. Furthermore, aplotaxene was present in the substrate in which these plants were growing. Aplotaxene inhibits growth of test species in soil. No other compounds were detected in our studies in sufficient quantity to be implicated in allelopathy.

Methods and Materials

Plant Material and Extraction *Carduus acanthoides* and *C. nutans* seeds were collected from wild populations in Pennsylvania located at GPS coordinates 40.820736, −77.847257 and 40.237254, −76.893675, respectively. Plants were grown in a greenhouse in Oxford, Mississippi, at ambient temperature, in a 50/50 mixture of Metro-Mix 350 (a greenhouse growth medium containing vermiculite, sphagnum peat moss, bark ash, nutrients, and dolomite limestone) (Sun Gro Horticulture, Agawam, MA 01001, USA) and soil conditioner (finely ground pine (*Pinus* spp.) bark from Sims Bark Co., Inc., Tuscumbia, AL 35674, USA) in 8.81-L pots from January to March, 2012. Fresh roots and shoots (almost all leaves) of rosette stage plants were air dried at room temperature in a fume hood for 48 h, providing 13.6 g of roots and 30.3 g of shoot material from *C. acanthoides* and 15.3 g of roots and 36.8 g of shoot material from *C. nutans*. After grinding in a Planetary Ball Mill Type PM 400 (Haan, Germany), each plant part was soaked in solvents of increasing polarity. Briefly, plant material was soaked at room temperature in methylene chloride (CH_2Cl_2), 95 % ethanol (EtOH/H_2O; v/v), and water, sequentially, for 24 h in each solvent followed by Buchner funnel filtration, rotary evaporation, and lyophilization. The yields of root and shoot extractables from *C. acanthoides* and *C. nutans* were calculated based on mass (Table 1).

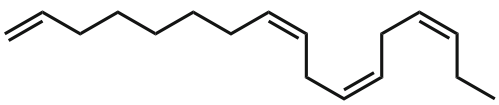

Fig. 1 Structure of aplotaxene

Gas Chromatography/Mass Spectrometry Analysis Samples were analyzed by GC/MS on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. The GC was equipped with a CP Sil 8 CB capillary column (30 m×0.25 mm, with film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 °C, column temperature, 60–240 °C at 3 °C/min then held at 240 °C for 5 min; carrier gas, He; injection volume, 1 μl (splitless). MS mass range from 40 to 650 *m/z*, filament delay of 3 min, target TIC of 20,000, a prescan ionization time of 100 μsec, an ion trap temperature of 150 °C, manifold temperature of 60 °C, and a transfer line temperature of 170 °C.

Phytotoxicity-guided Fractionation of C. acanthoides - Fractionation of the extracts was carried out with column chromatography using a Biotage, Inc. Horizon™ Pump (Charlottesville, VA, USA) equipped with a Horizon™ Flash Collector and fixed wavelength (254 nm) detector. Initially 371.3 mg of CH_2Cl_2 extractable material from the roots was separated on a Biotage 40+M column (normal phase 40–63 μm, 60 Å, 40 x 150 mm) running at 40 ml min^{-1} using a *n*-hexane:diethyl ether step gradient beginning with 100:0 to 50:50 over 1,600 ml followed by 50:50 to 0:100 over 600 ml. Fractions of 22 ml were collected and recombined, based on TLC similarities and UV chromatogram (254 and 280 nm) peak profiles, into 11 distinct fractions of 209.6 mg total (Table 1) and submitted for bioassay. Fraction A was identified as pure aplotaxene using ^{1}H NMR, ^{13}C NMR, and GC/MS. Spectroscopic data were in complete agreement with published values (Binder et al. 1992; Bohlmann and Abraham

Table 1 Bioassay-guided fractionation showing the amount of material recovered in each extract/fraction and its phytotoxicity[a]

Species	Part (g)	Extraction Solvent/Fraction	Weight (g)	Phytotoxicity (1 mg/ml)	
				Lettuce	Bentgrass
Carduus nutans	Roots (15.33)	CH_2Cl_2	0.420	0	1
		MeOH	1.242	0	0
		H_2O	0.0010	0	0
	Leaves (36.83)	CH_2Cl_2	0.812	0	0
		MeOH	2.718	0	0
		H_2O	0.004	0	0
Carduus acanthoides	Roots (13.58)	CH_2Cl_2	0.371	0	2
		MeOH	1.157	0	0
		H_2O	0.004	0	0
	Leaves (30.31)	CH_2Cl_2	1.444	0	0
		MeOH	2.182	0	0
		H_2O	0.004	0	0
Carduus acanthoides	Root Fractions (0.371)	Fraction A	0.059	1	4
		Fraction B	0.019	0	1
		Fraction C	0.003	0	0
		Fraction D	0.013	0	0
		Fraction E	0.005	0	3
		Fraction F	0.003	0	4
		Fraction G	0.014	0	3
		Fraction H	0.023	0	0
		Fraction I	0.020	1	0
		Fraction J	0.027	1	1
		Fraction K	0.024	1	1

[a] Values denote toxicity at 1.0 mg/ml to (lettuce, bentgrass) 0 = no effect, 5 = maximum effect

1981; Christensen 1992; Quintana et al. 2009), allowing the unequivocal identification of fraction A as aplotaxene ((Z,Z,Z)-heptadeca-1,8,11,14-tetraene).

GC-FID Percent Purity Analysis and Quantitative Analysis of Aplotaxene GC analyses were performed on fraction A (supplementary Fig. S-1) using a Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID). The GC was equipped with a CP Sil 8 CB capillary column (30 m×0.25 mm, with film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 °C, column temperature, 60–240 °C at 3 °C/min then held at 240 °C for 5 min; carrier gas, He; injection volume, 1 μl (splitless). Pure isolated aplotaxene described above was used as an external standard. With three concentration points (0.25, 0.50, and 1.0 mg ml^{-1}), an external standard least squares regression was performed for quantification. Linearity was imposed by using response factors and regression coefficients independently. Response factors were calculated using the Equation R.F.=PA/C, where PA was the detector response in peak area and C was the analyte concentration. The target peaks were confirmed by retention time. Confirmed integrated peaks were then used to determine the percentage of aplotaxene in the sample. The R.F. of the target chemical constituent was used to determine the "percent" for each sample using the equation:

$$(\mathrm{PA}/\mathrm{R.F.}/\mathrm{C}) \cdot 100 = \%.$$

Percent purity of fraction A was 100 % aplotaxene.

Determination of Aplotaxene in Wild C. acanthoides To determine the amount of aplotaxene in roots of wild *C. acanthoides*, rosette stage plants (mostly leaves) and flowering plants (leaves, stems, and flowers) were collected from a field in Pennsylvania at GPS coordinates 40.8234, −77.8771 and 40.8111, −77.8730, respectively, during early August of 2013. The roots from each plant stage were freeze-dried for 72 h and then ground separately in a Planetary Ball Mill Type PM 400, providing 6.75 g of roots from rosette plants and 49.00 g of roots from flowering plants. The root samples were extracted three consecutive times with excess CH_2Cl_2 at room temperature for 24 h, and the extracts were filtered using a Buchner funnel. The solvent was removed by rotary evaporation to obtain 124.2 mg of crude root extract from rosettes and 337.8 mg from flowering plants.

GC/FID analysis was performed as described in the above quantitative analysis. The FID peak areas were used to calculate the percentage of aplotaxene in each extract. Extraction yields were used to calculate the amount of aplotaxene per dry weight of roots.

Bioassays with Lactuca sativa and Agrostis stolonifera without Soil Silica gel column chromatographic fractionation of the CH_2Cl_2 extract of the leaves and roots of *Carduus acanthoides* and *C. nutans* was guided by lettuce (*L. sativa*) and bentgrass (*A. stolonifera*) bioassay according to (Dayan et al. 2000) in order to isolate phytotoxic fractions. A filter paper (Whatman #1) and 5 mg of *L. sativa* seeds or 10 mg of *A. stolonifera* seeds were placed in each well of a 24-well plate (Corning Inc., Corning, NY, USA). Test fractions were dissolved in acetone or water in a ratio of 1 to 10 (test material to solvent), depending on the solubility of extractables, to obtain stock solutions, which were then tested by adding 25 μl of the stock solution to 225 μl of distilled deionized (DDI) water to give a test concentration of 1 mg/ml. All samples including extract-free controls were adjusted to a final concentration of acetone of 3 %. Plates were covered, sealed with parafilm, and incubated at 26 °C in a Conviron growth chamber at 173 mol s^{-1} m^{-2} continuous photosynthetically active radiation (PAR). Phytotoxicity was qualitatively evaluated by visually comparing seed germination and seedling growth in each well with the extract-free 3 % acetone solution after 7 d. Phytotoxicity was estimated by using a rating scale of 0 to 5, where 0 = no effect and 5 = no germination of the seeds. Each experiment was repeated in triplicate. The same methodologies for growth and treatment were used for lettuce and bentgrass bioassays of aplotaxene, measuring the weight (lettuce), length of roots (lettuce) and shoot length (bentgrass) to determine the I_{50} values.

Cellular Leakage Bioassay Effects of aplotaxene on cellular leakage of cucumber (*Cucumis sativa* L.) cotyledon discs was determined by the method of Dayan and Watson (2011).

Bioassays of Aplotaxene in Soil The substrate used in this experiment was Commerce silty clay loam soil collected in a field that has never been treated with herbicides near the USDA Jamie Whitten Research Center in Stoneville, Mississippi (coordinates: 33° 25′ 22.68N 90° 53′ 52.9″W). Soil characteristics were as follows: fine silty, mixed, superactive, nonacid, thermic Fluvaquentic Endoaquept (Soil Survey Staff 2010), 38.5 % sand, 47.75 % silt, 13.75 % clay, organic matter 1.08 %, pH 6.4, 35 μg g^{-1} Mg, 8.2 μg g^{-1} K, and 92.8 μg g^{-1} Ca. The soil was air-dried, sieved (0.5 mm), and stored at room temperature.

Aplotaxene was dissolved in acetone and applied to dry soil to give the appropriate doses. Soil was dried in the lyophilizer for 5 min to remove acetone. Soil without aplotaxene application was used as a control. Twenty-four-well plates were used for each substrate, 12 for treated soil and 12 for untreated. Half of the wells were sown with lettuce and the other half with bentgrass. In each well, 0.2 ml of test solution were added to 0.6 g of soil. The amount of seeds used in each cell was approximately 5 mg of lettuce or 10 mg of bentgrass.

Plates were incubated at 26 °C in a Conviron growth chamber at 173 mol s^{-1} m^{-2} PAR. To maintain the humidity, the trays were covered with plastic chambers. Shoot weights were determined 7 d after planting.

Recovery of Aplotaxene from Soil One gram of Bosket very fine sandy loam soil, nearly level phase, from Washington County, MS, USDA which had been dried by lyophilization was added to 20 ml scintillation vials. A 10 mg/ml stock solution of aplotaxene in acetone was made and labeled Stock A and a 1:1000 dilution of stock A was made and labeled Stock B. In triplicate, 400 μL of stock A solution was added to vials containing 1 mg soil, and the acetone removed in the lyophilizer for 5 min, yielding 4 mg of aplotaxene per gram of soil. Analogously, 400 μl of stock B was added and the acetone removed in the lyophilizer leaving 4 μg of aplotaxene per gram of soil. Each sample was extracted into 3 ml of CH_2Cl_2, and solvent was removed under nitrogen. The residue was re-dissolved in 1 ml of acetone and analyzed as described above for quantitative analysis of aplotaxene in soil.

Bioassays with Duckweed (Lemna paucicostata) Bioassays were performed as previously described in detail (Michel et al. 2004). Briefly, *Lemna paucicostata* stocks were grown from a single colony consisting of a mother and two daughter fronds in a beaker on modified Hoagland media containing 1,515 mg L^{-1} KNO_3, 680 mg L^{-1} KH_2PO_4, 492 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 20 mg L^{-1} Na_2CO_3, 1,180 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mg L^{-1} $MnCl_2$, 0.025 mg L^{-1} $CoCl_2$, 0.025 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and 18.355 mg L^{-1} Fe-EDTA. The media was adjusted to pH 5.5 with 1 M NaOH and filtered through a 0.2 μm filter. Each well of a nonpyrogenic polystyrene sterile 6-well plate (CoStar 3506, Corning Inc., Corning, NY, USA) was filled with 4,950 μl of the Hoagland media mixed with 50 μl of acetone or 50 μl of acetone with the appropriate concentration of aplotaxene. Independent experiments demonstrated that there was no effect of 1 % acetone on growth of the plants. Two three-frond colonies from 4- to 5-d-old stock cultures were placed in each well. Total frond area per well was recorded by the image analysis system, Scanalyzer (LemnaTec, Würselen, Germany) at days 4 and 7. Percent increase at days 4–7 was determined relative to baseline area at day 0.

Aplotaxene Content of Sand in which C. acanthoides is Grown Sand was collected in June, 2012 from 3.79-L- pots

in which *C. acanthoides* had been grown in the greenhouse for 45 d. The sand was collected from under the growing plants by lifting the soil/plant mass and gently shaking while loose soil was collected. All sand from three pots was collected and dried in a lyophilizer for 3 d, providing 3.6, 3.9, and 3.8 kg of sand. The control sample, sand without a plant, weighed 0.7 kg. The sand samples were extracted with CH_2Cl_2 (2 L or 1 L for control) while stirring for 2 h, filtered and concentrated. Solutions of one and 10 mg ml^{-1} of the samples were placed into a 2-ml GC vial and analyzed as described above by GC/FID quantitative analysis.

Results

Bioassay-Guided Fractionation of C. nutans and C. acanthoides Only the CH_2Cl_2 extracts of the roots of the two species possessed phytotoxicity at 1 mg ml^{-1} (Table 1). Upon further fractionation of the CH_2Cl_2 extract with column chromatography, fraction A had the highest phytotoxicity for both test species, as well as the highest yield of material (Table 1). GC and TLC analyses indicated that fraction A contained one major compound (Fig. S-1). Similar fractions of roots and leaves of both *Carduus* species indicated that the compound is common to both species and is present only in the roots (Fig. S-1).

Identification of Aplotaxene ^{1}H NMR, ^{13}C NMR, and GC/MS studies were conducted on the compound in fraction A. It was identified as aplotaxene (Fig. 1) according to the criteria of Binder et al. (1992); Bohlmann and Abraham (1981); Christensen (1992); and Quintana et al. (2009). By dry weight, the roots of greenhouse-grown *C. acanthoides* contained about 0.44 % of this compound. Roots of field-grown *C. acanthoides* in the rosette and flowering stages contained 0.03 and 0.008 % aplotaxene, respectively. Both of these values were considerably less than the 0.44 % aplotaxene measured in roots of greenhouse-grown plants.

Phytotoxicity of Aplotaxene Aplotaxene was weakly toxic to lettuce in a Petri dish assay without soil, with I_{50} concentrations after 7 d of exposure for fresh weight and root length inhibition of 2.8±1.0 and 2.3±0.5 mM, respectively (Fig. 2). There was no effect at 1 mM, and complete inhibition at 10 mM. The I_{50} concentration for bentgrass shoot length was 4.2±2.0 mM, with no effect at 1 mM and complete inhibition at 10 mM (Fig. 3). Aplotaxene was moderately phytotoxic to *L. paucicostata*, having no effect at 0.1 mM or below, but almost completely inhibiting growth at 1 mM, with an I_{50} value of less than 0.333 mM (Fig. 4). The dose/response relationship was the same at 4 and 7 days after exposure, indicating there was no dissipation of phytotoxicity over a 1-week period. Aplotaxene caused cucumber cotyledon discs to leak electrolytes at both 0.3 and 3 mM, with leakage reaching almost the maximum possible value at 3 mM after a 24 h exposure (16 h dark, followed by 8 h light) (Fig. 5).

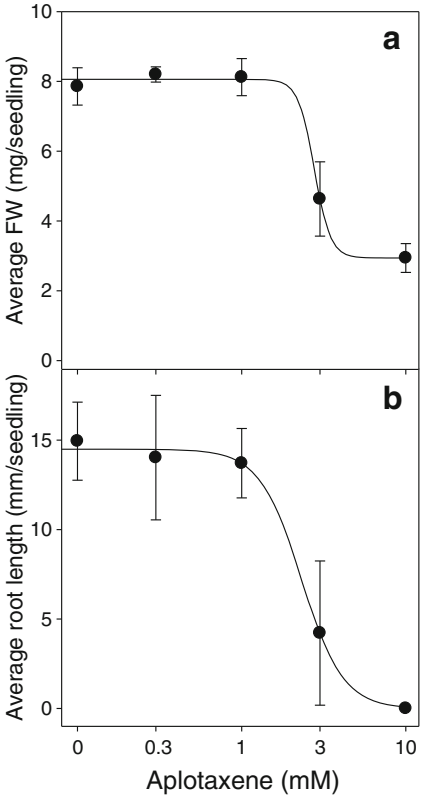

Fig. 2 Effects of aplotaxene on A) seedling FW and B) root length of lettuce grown on filter paper at 7 d after planting. Error bars are one standard deviation

Aplotaxene was active in soil, as evidenced by its strong inhibition of lettuce fresh weight and longitudinal root growth at 1 mg of aplotaxene per gram of dry soil (Fig. 6). At 3 mg per gram of soil, seedling growth and development was

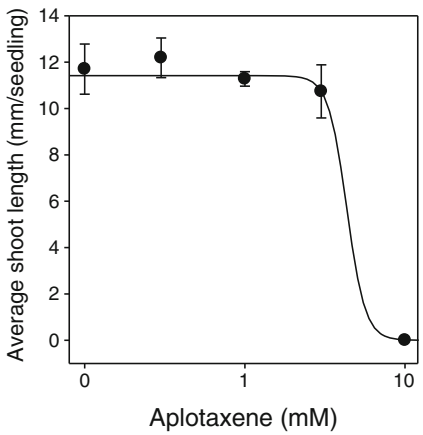

Fig. 3 Dose–response of effect of aplotaxene on bentgrass growth after 7 d of exposure. Error bars are one standard deviation

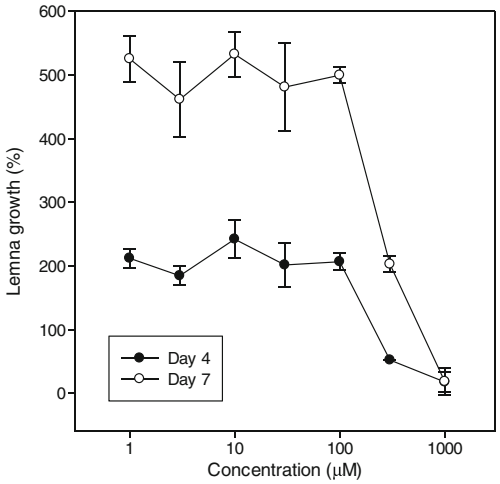

Fig. 4 Dose–response of effect of aplotaxene on *Lemna paucicostata* growth after 4 and 7 d of exposure. Growth is presented as a percent of the frond area at the beginning of the treatment. To allow depiction on a log scale, the solvent control values were plotted at 1 μM. Error bars are one standard error

completely inhibited. The I_{50} values for fresh weight and longitudinal root growth were 0.63 and 0.49 mg g^{-1}, respectively. The relative sensitivity of bentgrass compared to lettuce in soil was similar to that observed without soil (Figs. 2 and 3), with an I_{50} value of 2.56 mg g^{-1} for shoot length inhibition (Fig. 7).

Aplotaxene in the Rhizophere of C. acanthoides A soil spiking study was conducted to calculate the recovery of aplotaxene from soil. Dried soil samples containing 4 mg g^{-1} soil and 4 μg g^{-1} aplotaxene were extracted, resulting in 81.0±31.6 and 71.3±18.5 % recoveries.

The CH_2Cl_2 extract of three different pots in which *C. acanthoides* was grown for 45 days in the greenhouse had an average yield of aplotaxene of 3.63±0.49 μg g^{-1} of

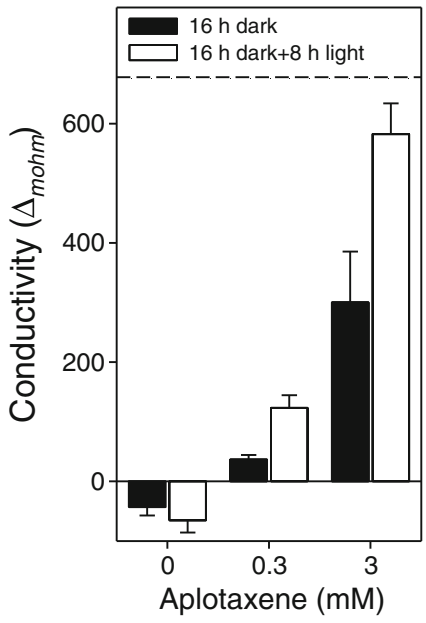

Fig. 5 Effects of two different concentrations of aplotaxene on electrolyte leakage from cucumber cotyledon discs under different dark and light exposures. The dotted line is the maximum level of leakage possible. Error bars are one standard deviation

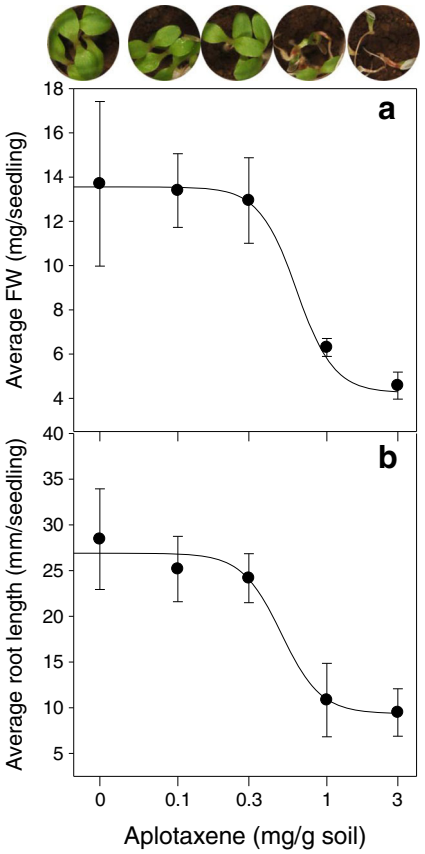

Fig. 6 Effects of aplotaxene on **a**) seedling FW and **b**) root length of lettuce in soil at 7 d after planting. Error bars are one standard deviation

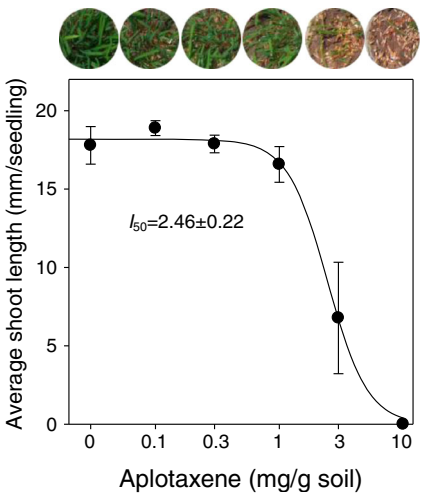

Fig. 7 Effects of aplotaxene on growth of bentgrass in soil at 10 d after planting. Error bars are one standard deviation

sand. The spiking recovery study with ca. 71–81 % recovery indicates that the levels of aplotaxene from soil in which the plants had grown were probably 4.5 to 5.1 μg g^{-1} of sand.

The chromatograms of the aplotaxene standard (A), the sample from a pot yielding 3.3 μg g^{-1} of sand (B), and an extract from the control sand (C) are shown in Fig. 8. The main peak at 34.8 min is aplotaxene, with several minor peaks even in the aplotaxene standard (Fig. 8a). These were apparently oxidation products of aplotaxene,suggesting that the compound is somewhat unstable.

Discussion

The weak phytotoxicity of aplotaxene in soil seems not to support the view that this compound acts as an allelochemical. Indeed, Quintana et al. (2009) claimed that aplotaxene is not phytotoxic at concentrations up to 2.15 mM. This is contrary to our bioassays in which lettuce growth was inhibited almost 50 % and duckweed growth was completely inhibited at a comparable concentration (Figs. 2, 4). However, aplotaxene might have been oxidized in their bioassay, which involved treating and growing the test plants in liquid culture on a rotary shaker. The 1,4-dienes found in the aplotaxene molecule may be oxidized by air in a manner analogous to the oxidation of unsaturated fatty acids such as linoleic acid and oleic acid, which also contain 1,4-dienes and are easily oxidized by exposure to air (Farag et al. 1989).

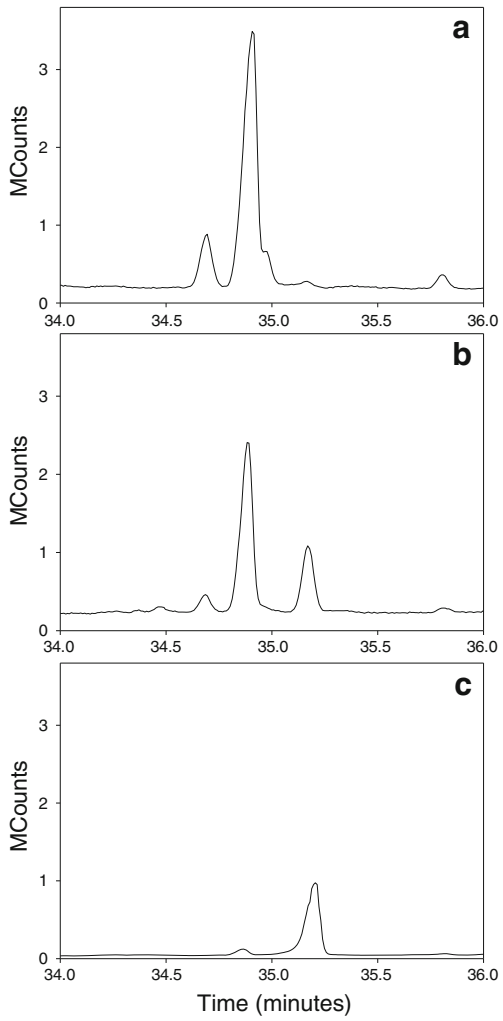

Fig. 8 Gas chromatography/mass spectrometry (GC/MS) analysis of aplotaxene standard (**a**), the extract from sand in which *Carduus acanthoides* had been grown (**b**), and the control extract from sand without plants (**c**)

Previous work with *Carduus* spp. did not reveal aplotaxene. However, Wardle et al. (1996) only examined aqueous extracts. Formisano et al. (2007) looked for lipophilic allelochemicals from a *Carduus* species but only considered the shoots, which do not produce aplotaxene (Fig. S-1).

The weak phytotoxicity of aplotaxene would eliminate it as a possible allelopathic compound if it were not for the relatively high level of the compound in the roots and the fact that its activity is not greatly decreased by soil. The factors of phytotoxicity in the soil and the amount produced by the plant can be used to calculate the total activity of the compound as an allelochemical (Hiradate 2006; Hiradate et al. 2010). To compare potential contributions of different compounds to allelopathy, the molar concentration of the compound in the donor plant tissue is divided by the specific activity of the compound in soil to generate a total activity, a unitless parameter. Using values for concentration of aplotaxene in roots of greenhouse-grown plants (20 mM) and the EC_{50} for lettuce (6.5 mM), the total activity for aplotaxene from *C. acanthoides* is 3.08 with lettuce as a test species. The total activity against lettuce calculated by Hiradate et al. (2010) for the allelochemicals juglone (from *Juglans* spp.), L-mimosine (from *Leucaena leucocephala*), (+)-catechin (from *Fagopyrum esculentum*), *trans*-cinnamic acid (from *Xanthium occidentale*), and *cis*-cinnamic acid (from *Brassica parachinensis*) were 3.3, <2, <0.05, 0.25, and 0.057, respectively, in volcanic ash soil and 6.8, <2, <0.05, 0.25, and 0.4, respectively, in alluvial soil. Some of these compounds (e.g., *cis*-cinnamic acid) are quite phytotoxic in the absence of soil, but are much less active in some soils. A total activity value of 3.08 is in the range of these established allelochemicals, thus supporting the view that aplotaxene could play a role in allelopathy of *C. acanthoides*. However, the total activity values for aplotaxene in *C. acanthoides* are only 0.21 and 0.011 based on levels of the compounds in the roots of field-collected plants. Whether the differences in aplotaxene content were due to differences between greenhouse and field environments could not be determined from our experiments, as the plants were sampled at different developmental stages.

The level of aplotaxene in the roots of *Carduus* species could vary with developmental stage and/or stress conditions. Yano et al. (1983) found little or no variation in aplotaxene

content of *Cirsium japonicum* root oil between different developmental stages of the plant, but noted that levels of aplotaxene derivatives were considerably lower 2 to 3 months before flowering. In our study, roots of plants from the field had more than twice the concentration of aplotaxene in the rosette stage than during the flowering stages of development, indicating that this species may be more allelopathic in early stages of development. Differences in allelopathy at different stages may be related to differences in morphology; rosettes are low and flat to the ground, and these early stages suffer significantly from competition for light and space with existing vegetation (Rauschert and Shea 2012), while flowering plants can be >1 m tall (Skarpaas and Shea 2007) and consequently are less affected by surrounding vegetation. Our limited data indicate that aplotaxene levels diminish as the plant develops.

The levels of aplotaxene measured in soil in which the *C. acanthoides* had been growing were much lower than levels needed to inhibit growth in bioassays in soil. However, total aplotaxene of the soil in the pot was analyzed with no attempt to assess concentration gradients related to distance from the root tissue. Rhizophere aplotaxene levels might have been much higher. Furthermore, loss of volatile aplotaxene or oxidation during sample preparation may have reduced the amount measured. In addition, the amount of aplotaxene in soil at any point in time does not measure the flux of aplotaxene that might occur in soil. A continual movement from roots to soil, where the compound may be lost via processes such as oxidative degradation is a quite different situation from an artificial system in which soil is spiked with a fixed amount of compound. Furthermore, it is possible that oxidized products and other derivatives of aplotaxene could be more phytotoxic than the parent compound. Numerous derivatives of aplotaxene have been described in other species (e.g., Christensen 1992; Nazaruk et al. 2012).

Aplotaxene has been identified as a root and/or rhizome constituent of species including *Cirsium* spp. (Christensen 1992; Nazaruk et al. 2012), *Rhaponticum carthamoides* (Havlik et al. 2009), *Inula racemosa* (Bokadia et al. 1986), *Saussurea* (formerly *Aplotaxus*) *lappa* (Choi et al. 2009), and *Centaura* spp. (Cossy and Aclinou 1990; Quintana et al. 2009; Tesevic et al. 1994, 2003). As much as 80 % of the volatile oil from roots of *Cirsium dipsacolepis* is aplotaxene and four of its derivatives (Takano and Kawaminami 1988). Aplotaxene accounts for 75 % of the essential oil of *Cirsium japonicum* root (Miyazawa et al. 2003), and 20 % of costus oil extracted from *Aplotaxis* (now *Saussurea*) *lappa* (Naves 1949). French et al. (1988) reported that aplotaxene made up 0.032 % of the fresh weight of Canada thistle (*Cirsium arvense*), and noted that aplotaxene is a fungal spore germination stimulant for the teliospores of Canada thistle rust (*Puccinia punctiformis*), but functions for root aplotaxene are not known. Some aplotaxene-producing species, such as *Cirsium* spp., *Inula* spp., and *Centauria* spp. are reported to be allelopathic (e.g., Chon et al. 2003; Hierro and Callaway 2003; Khan et al. 2009), but the active allelochemical(s) have not been identified in any of these genera.

Our data support the hypothesis that aplotaxene is involved in allelopathy of *Carduus* species. In our studies, bioassay-directed isolation of phytotoxins from roots of two *Carduus* species found only soil-active aplotaxene to be produced in sufficient quantities to act as an allelochemical. Additional studies are required to evaluate whether aplotaxene has a similar role in other species that produce this lipophilic compound in the roots.

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