J Chem Ecol (2008) 34:499–507
DOI 10.1007/s10886-008-9449-8

Bidens pilosa L. Exhibits High Sensitivity to Coumarin in Comparison with Three Other Weed Species

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Received: 11 September 2007 / Revised: 8 February 2008 / Accepted: 11 February 2008 / Published online: 13 March 2008

Abstract Nine natural plant compounds were screened for phytotoxicity to *Bidens pilosa* L. a troublesome weed in field and plantation crops. The sensitivity of three other weed species to coumarin, the most active identified compound, was also evaluated. Coumarin, at a concentration of 500 μM, had little effect on germination and growth of *Senna obtusifolia* L., *Euphorbia heterophylla* L., and *Ipomoea grandifolia* L. when compared with its effects on *B. pilosa* L. In a concentration range of 10–100 μM, coumarin caused a dose-dependent inhibition of germination and growth of *B. pilosa* L. The measurements of some parameters of energy metabolism revealed that coumarin-treated root tissues exhibited characteristics of seedlings in an earlier stage of growth, including higher respiratory activity and higher activities of alcohol dehydrogenase and lipoxygenase. These results suggest that coumarin inhibition of germination and growth of *B. pilosa* L. was not a consequence of an impairment of energy metabolism. Rather, it seems to act as a cytostatic agent, retarding germination. At concentrations above 50 μM, coumarin increased lipoxygenase activity and the level of conjugated dienes of root extracts, suggesting that it may induce oxidative stress in seedling roots.

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Keywords *Bidens pilosa* L. · *Asteraceae* · Seed germination · Seedling growth · Respiratory energy metabolism · Alcohol dehydrogenase · Lipoxygenase · Coumarin

Abbreviations

AOX	alternative oxidase
COX	cytochrome oxidase
DTT	dithiothreitol
EDTA	ethylene diamide tetracetic acid
KCN	potassium cyanide
MDA	malondialdehyde
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid

Introduction

Bidens pilosa L., an annual native of tropical America, is a dicot weed of the *Asteraceae* family. The species is a troublesome weed in field and plantation crops in more than 40 countries (Holm et al. 1977). In Brazil, it is responsible for yield losses of several crops, particularly soybean. It was demonstrated that *B. pilosa* L. first evolved resistance to herbicides known as acetolactase synthase inhibitors in 1993, and there were estimates that the resistant biotype continues to increase its distribution and prevalence (Christoffoleti and Foloni 1999). Because of increasing incidence of weeds evolving resistance to many commercial herbicides, there is growing interest in the development of alternative methods for weed control based on natural products. Plants produce thousands of secondary products that represent a large reservoir of novel chemical structures with biological activity. The use of plant species

with weed-suppressing ability thus has been considered for biological weed management in crop production (Putnam and Duke 1974; Macías 1995; Wu et al. 1999; Duke et al. 2000; Vyvyan 2002; Khanh et al. 2006). Numerous secondary plant products of the phenolic, flavonoid, and terpenoid classes have been implicated as compounds responsible for plant growth suppression (Reigosa et al. 1999; Vaughn and Spencer 1993; Dudai et al. 1999; Duke et al. 2000; Chon and Kim 2004; Kohli et al. 2006). However, there are fewer studies that are concerned with modes of action. Mitochondrial respiratory metabolism is essential to produce energy and precursors for biosynthesis of new cellular structures. An effect on respiratory metabolism could be a mode of action of natural compounds in suppressing the germination and growth of weeds.

In view of this, the aim of the present work was to identify which among nine compounds exerts high and selective phytotoxicity on *B. pilosa* L. We chose representative compounds of the phenolic and terpenoid classes, namely, caffeic, *p*-coumaric, ferulic, protocatechuic, and vanillic acids, coumarin, flavone, camphor, and eucalyptol. The effects of coumarin, the most active identified compound, on biochemical processes, critical for seed germination and seedling growth, were also evaluated. For the latter purpose, respiratory activity and the activity of alcohol dehydrogenase (EC 1.1.1.1) in seedling roots during postgerminative growth were measured. Some parameters of oxidative stress were also evaluated, including lipoxygenase activity (EC 1.13.11.12) and the content of malondialdehyde and conjugated dienes.

Methods and Materials

Reagents Camphor, caffeic acid, eucalyptol, *p*-coumaric acid, coumarin, ferulic acid, flavone, protocatechuic acid, vanillic acid, NAD^+, linolenic acid, and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reagents were of the purest grade available.

Seed Germination and Growth Seeds of *B. pilosa* L., *Euphorbia heterophylla* L., *Senna obtusifolia* L., and *Ipomoea grandifolia* L. were purchased from a commercial supplier (Cosmos Agrícola Produtos e Serviços Rurais Ltda, Brazil). Seeds were surface-sterilized in a 1.0% sodium hypochlorite solution. After washing in distilled water, seeds were placed on a double sheet of germination paper in plastic germination boxes (gerbox; 110×110 mm), moistened with 5–12 ml of distilled water or plant natural compound solutions at a concentration of 500 μM. Ferulic, caffeic, vanillic, and protocatechuic acids were dissolved in distilled water. Camphor, eucalyptol, flavone, and coumarin were dissolved in a 0.1% dimethylsulfoxide solution. Flavone was assayed at a concentration of 250 μM because of its low solubility in the 0.1% dimethylsulfoxide solution. Coumarin was assayed in a concentration range of 10 to 100 μM. Controls were performed to exclude the interference of dimethylsulfoxide, but no significant changes in seedling growth were found. Each treatment was applied to three plates (replicates), and each replicate consisted of 50 seeds distributed over gerbox. Experiments were repeated four to six times. Boxes were placed in a growth chamber programmed for the following regime for *B. pilosa* L.: 8/16 hr L/D, 30°/20°C. *E. heterophylla* L. seeds were allowed to germinate and grow at 25°C and on a 12/12 hr L/D photoperiod. The regimes for *S. obtusifolia* L. and *I. grandifolia* L. were 30°C and 12/12 hr L/D photoperiod. The photon flux density of the growth chamber was approximately 230 μmol m^{-2} s^{-1} photon flux. A seed was considered germinated when the radicle was 2.0 mm or longer. Seeds that had germinated at 2, 4, or 6 d were selected for growth tests. Seedlings were removed, dried on filter paper, and the primary roots were excised for measurements of their length and fresh weight. Data were expressed as centimeters or milligrams per root. The mean germination time was calculated according to Eq. 1 (Labouriau and Osborn 1984):

$$\bar{t} = \sum n_i t_i \Big/ \sum n_i \qquad (1)$$

$\bar{t}$ Mean germination time
n Number of germinated seeds between the times t_{i-1} and t_i

Respiration of Excised Primary Roots Oxygen consumption of primary roots from *B. pilosa* L. seedlings was measured polarographically at 25°C with a Clark-type electrode positioned in a closed plexi-glass chamber. Primary roots were removed from seedlings and rinsed in distilled water. For each measurement, samples of six roots were cut into segments 5–10 mm long as measured from the growth apex, weighed, and placed immediately in the oxygen electrode vessel that contained 2 ml of nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$, and 20 μM NaH_2PO_4 (Larkin 1987). For estimating the contribution of the mitochondrial cytochrome oxidase (COX; KCN-sensitive respiration) and mitochondrial alternative oxidase (AOX) plus extramitochondrial oxidases (KCN-insensitive respiration) to the overall O_2 uptake, 270 μM potassium cyanide (KCN) were added to the reaction medium. Oxygen uptake was monitored for 12–15 min. Uptake rates were calculated from the polarographic records considering an initial concentration of dissolved oxygen of 240 μM at

25°C (Estabrook 1967) and referred to the fresh weight of the roots.

Alcohol Dehydrogenase Activity Alcohol dehydrogenase activity was assayed in root extracts from seedlings grown in the absence of coumarin for 2, 3, or 4 d or in the presence of coumarin (10–50 μM) for 4 d. Primary roots (approximately 0.2 g fresh weight) were excised from the seedlings, weighed, and transferred to a mortar, thoroughly mixed with 3.0 ml of a medium that contained 50 mM Tris–HCl (pH 7.4), 1.0 mM ethylene diamide tetracetic acid, and 2.0 mM dithiothreitol. Extracts were centrifuged for 20 min at 20,000×*g* and 5°C. The supernatant was decanted and used as the enzyme source. Alcohol dehydrogenase activity was measured according to Lee (1982). The reaction medium contained 50 mM Tris–HCl (pH 7.4), 1.0 mM NAD^+, and 200 μl of enzyme extract. The reaction was initiated by the addition of 120 μM *n*-propanol. Enzyme activity was evaluated as the initial rate of NAD^+ reduction, which was calculated from the increase in absorbance at 340 nm. Enzyme activity was expressed as μmol min^{-1} (g fresh weight)$^{-1}$.

Lipoxygenase Activity Lipoxygenase activity was assayed in the root extracts from seedlings grown in the absence of coumarin for 2, 3, or 4 d or in the presence of coumarin (10–50 μM) for 4 d. Primary roots (approximately 0.2 g fresh weight) were weighed and transferred to a mortar and thoroughly mixed with 1.5 ml of a cold 50 mM K-phosphate (pH 7.0) solution containing 0.1% Triton X-100 (*v*/*v*). Extracts were centrifuged for 10 min at 12,000×*g* and 5°C. The supernatant was decanted and used as the enzyme source. Lipoxygenase was measured polarographically with a Clark-type oxygen electrode according to Siedow and Girvin (1980). The reaction medium contained 200 mM K-phosphate (pH 7.0) and 200 μl of enzyme extract. The reaction was initiated by the addition of linolenic acid (3.0 mM final concentration), dissolved in Tween 20. Oxygen uptake was monitored for 12–15 min, and the enzyme activity was expressed as μmol O_2 min^{-1} (g root fresh weight)$^{-1}$. Controls were run to exclude solvent effects.

Lipid Peroxidation Products The level of lipid peroxidation in primary root extracts was measured in terms of malondialdehyde (MDA) and conjugated diene contents. Approximately 200 mg of excised roots were homogenized in 4.0 ml of 96% (*v*/*v*) ethanol. The content of malondialdehyde (MDA) was assayed in 3.0 ml of the homogenate (Heath and Packer 1968). An equal volume of 10% trichloroacetic acid that contained 0.5% TBA was added to the homogenate. The mixture was heated to 95°C for 30 min and cooled quickly in an ice bath. After centrifuging at 10,000×*g* for 10 min, absorbance of the supernatant at 532 nm was read. The value for nonspecific absorbance at 600 nm was subtracted. The concentration of MDA was calculated by using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol (g root fresh weight)$^{-1}$.

For the conjugated diene measurement, a 1.0-ml aliquot of homogenate was mixed into an equal volume of 96% ethanol and centrifuged at 12,000×*g* for 10 min (Boveris et al. 1980). Absorbance of the supernatant was read at 234 nm, and the nonspecific absorbance at 500 nm was subtracted. Concentration of the conjugated dienes was calculated by using the extinction coefficient of 2.65×10^4 M^{-1} cm^{-1} and expressed as μmol (g root fresh weight)$^{-1}$.

Statistical Analysis The data shown in the graphs and tables were expressed as means±standard errors (SEM) of independent preparations. Data were analyzed with Student's *t* test or analysis of variance (ANOVA), significant differences between means being identified by Duncan's test. The comparisons are given in the text as probability values (*P*). $P\leq0.05$ being adopted as the minimum criterion of significance. The ID_{50} was computed by numerical interpolation by means of a cubic spline function. Statistical analyses were performed by using the Statistica™ software package.

Results

Effects on Germination and Growth The present work revealed different effects and potencies for each tested compound on *B. pilosa* (Table 1). Germination and seedling growth inhibition among the cinnamic acid derivatives varied greatly. At 500 μM, ferulic acid reduced germination to 42% of the control at the second day, but when germination occurred, the lengths and the fresh weights of the seedling roots were not modified. After 2 days, no significant modifications were observed. Vanillic acid, in contrast, did not affect seed germination, but reduced the growth of seedling roots at the fourth day. Caffeic, *p*-coumaric, and protocatechuic acids were inactive.

The actions of monoterpenes were also variable. Whereas eucalyptol was inactive, camphor inhibited both germination and growth of *B. pilosa*. At 500 μM, it reduced germination by 81% and 40% on the second and fourth days, respectively. The lengths and the fresh weights of the seedling roots were 39% and 51% reduced, respectively, on the second day. On the fourth day, the corresponding values were 19% and 11%.

Flavone, at 250 μM, had weak activities on *B. pilosa*. Germination was 13% reduced at day 4, but the lengths and

Table 1 Germination percentage, length, and fresh weight of primary roots of *B. pilosa* L. incubated for 2 or 4 d in water (control), in 1% (*v*/*v*) DMSO, in 250 μM flavone or in 500 μM caffeic, *p*-coumaric, ferulic, protocatechuic and vanillic acids, camphor, eucalyptol, and coumarin

Compound (500 μM)	Germination (%)		Root Length (cm)		Root Fresh Weight (mg per root)	
	Growth Period (d)					
	2	4	2	4	2	4
Control (*N*=19)	25.7±3.10	77.43±1.56	0.66±0.02	1.12±0.04	1.27±0.07	2.4±0.10
DMSO 1% (*N*=8)	19.2±2.33	72.80±2.80	0.64±0.04	1.07±0.05	1.18±0.09	2.24±0.10
Caffeic acid (*N*=6)	28.0±3.10	82.0±4.60	0.74±0.04	1.14±0.10	1.28±0.05	2.44±0.16
Coumaric Acid (*N*=4)	23.0±5.70	80.0±7.50	0.61±0.05	0.93±0.07	1.15±0.11	2.36±0.18
Ferulic acid (*N*=6)	10.67±2.29*	73.0±4.75	0.62±0.07	0.95±0.06	1.10±0.17	1.64±0.07*
Protocatechuic acid (*N*=4)	26.0±3.16	84.50±5.50	0.60±0.02	1.15±0.09	1.13±0.04	2.34±0.23
Vanillic acid (*N*=4)	15.5±4.79	64.0±12.36	0.59±0.03	0.69±0.09*	1.09±0.03	1.72±0.32*
Camphor (*N*=5)	3.60±0.98*	44.0±3.52*	0.39±0.11*	0.87±0.06*	0.58±0.26*	1.99±0.08
Eucalyptol (*N*=5)	21.2±1.63	70.80±3.93	0.73±0.01	1.16±0.06	1.19±0.10	2.27±0.17
Flavone (*N*=5)	20.8±2.87	63.2±3.01*	0.66±0.05	1.05±0.05	1.38±0.17	2.28±0.19
Coumarin (*N*=5)	0.0*	0.0*	–	–	–	–

Values are expressed as mean ± SE. Significant differences between treated seeds and the respective controls are indicated.
*$P \leq 0.05$, ANOVA with Duncan's multiple range test.

the fresh weights of the seedling roots of germinated seeds were not modified.

Coumarin was the most phytotoxic among all assayed compounds. At the 500 μM concentration, it completely suppressed germination of *B. pilosa*.

Effects of Coumarin on Germination and Growth of E. heterophylla, I. grandifolia, and S. obtusifolia To evaluate whether the phytotoxicity of coumarin is species specific, the effects of 500 μM coumarin on germination and growth of *I. grandifolia*, *S. obtusifolia*, and *E. heterophylla* were examined (Table 2). Germination of *S. obtusifolia* was completely inhibited by the second day, but by the fourth day, the number of germinated seeds was not different from that of the control. No significant modification was found in the germination of *E. heterophylla* and *I. grandifolia*, although coumarin affected seedling growth. The lengths of primary roots of *E. heterophylla* and *S. obtusifolia* were reduced without significant reduction in fresh weights, whereas in *I. grandifolia*, a reduction in both growth parameters was observed by the second day. Comparison among species reveals that coumarin was more phytotoxic

Table 2 Germination percentage, length, and fresh weight of primary roots of *E. heterophylla* L., *I. grandifolia* L., and *S. obtusifolia* L. incubated for 2 or 4 d in 1% (*v*/*v*) DMSO or in 500 μM coumarin

Plant species	Condition	Germination (%)		Root Length (cm)		Root Fresh Weight (mg per root)	
		Growth Period (d)					
		2	4	2	4	2	4
Euphorbia heterophylla	Control (*N*=4)	45.60±3.43	54.67±3.92	1.06±0.10	3.80±0.21	5.90±0.53	24.19±2.43
	Coumarin 500 μM (*N*=4)	34.67±4.37	51.50±3.78	0.28±0.01* (−74%)	1.47±0.06* (−61%)	4.52±1.04	22.74±0.20
Ipomoea grandifolia	Control (*N*=4)	13.4±1.54	22.2±0.49	0.51±0.05	1.98±0.17	4.00±0.33	10.34±0.8
	Coumarin 500 μM (*N*=4)	10.4±0.98	17.4±1.25	0.29±0.02* (–43%)	0.75±0.04* (–63%)	2.92±0.16* (−27%)	10.01±0.36
Senna obtusifolia	Control (*N*=5)	6.20±0.58	18.4±2.1	0.35±0.04	0.69±0.024	4.48±0.32	10.53±0.39
	Coumarin 500 μM (*N*=5)	0±0* (−100%)	12.6±0.81	0±0* (−100%)	0.41±0.03* (−41%)	0±0* (−100*)	10.4±0.76

Values are expressed as mean±SE. Significant differences between treated seeds and the respective controls are indicated.
*$P \leq 0.05$, ANOVA with Duncan's multiple range test.

to *I. grandifolia* than to *E. heterophylla* and *S. obtusifolia*. However, the phytotoxicity of coumarin on *B. pilosa* was significantly higher (at the same concentration [500 μM] ,it completely suppressed germination of this weed [Table 1]). This finding led us to perform a more extensive investigation of coumarin effects on *B. pilosa*.

Effects of Coumarin on Germination and Growth of B. pilosa When *B. pilosa* seeds were incubated in the presence of coumarin in the concentration range of 10–100 μM for 6 d, both seed germination and seedling root growth were significantly inhibited (Fig. 1). At d 2, complete suppression of seed germination was observed with coumarin at the 50 μM concentration or higher (Fig. 1a). The calculated ID_{50} were 10.6±2.1 μM, 23.8±4.5 μM, and 21.3±4.4 μM for germination, root length, and root fresh weight, respectively. At subsequent periods (d 4 and 6), germination and root growth were also reduced, though to lesser degrees. The ID_{50} for germination was increased to 46.5± 2.7 and 89.9±2.1 μM on the fourth and sixth day of incubation, respectively. Mean germination time increased from 88.4±4.5 hr in the control to 94.0±2.7, 105.8±2.6, 122.8±1.1, and 129.1±2.8 hr with 10, 25, 50, and 100 μM coumarin, respectively. Root fresh weight was reduced to a lesser extent compared to reduction of root length. At d 6, for example, coumarin up to 50 μM had not affected root fresh weight, while an inhibitory action on root length was observed with 10 μM coumarin.

After 4 d of incubation, the first leaves were visible, indicating that photosynthesis had started to contribute to seedling energy metabolism. Thus, the subsequent experiments were performed with seedlings grown for a maximum of 4 d, assuring that the contribution of mitochondrial respiration to seedling energy metabolism was predominant.

Effects of Coumarin on Respiratory Activity of Excised Primary Roots and on Activities of Alcohol Dehydrogenase and Lipoxygenase in Primary Root Extracts Respiratory activity of primary roots and activity of alcohol dehydrogenase and lipoxygenase in the control series (absence of coumarin) were measured in seedlings grown for 2, 3, and 4 d. As shown (Fig. 2), all these parameters were higher shortly after the emergence of primary roots (d 2) and decreased progressively during the subsequent growth period. From the second to the fourth day, the overall O_2 consumption rates were reduced by 40% on a fresh-mass basis. The relative contribution of the KCN-sensitive respiration to the overall respiration decreased from 73.9% at d 2 to 68.9% at d 4. The decline in the activities of alcohol dehydrogenase and lipoxygenase during the growth period was more accentuated (Fig. 2). At d 3 and 4, alcohol dehydrogenase activity was, respectively, 33.2% and 8.6% of that one found on d 2. Lipoxygenase activity decreased

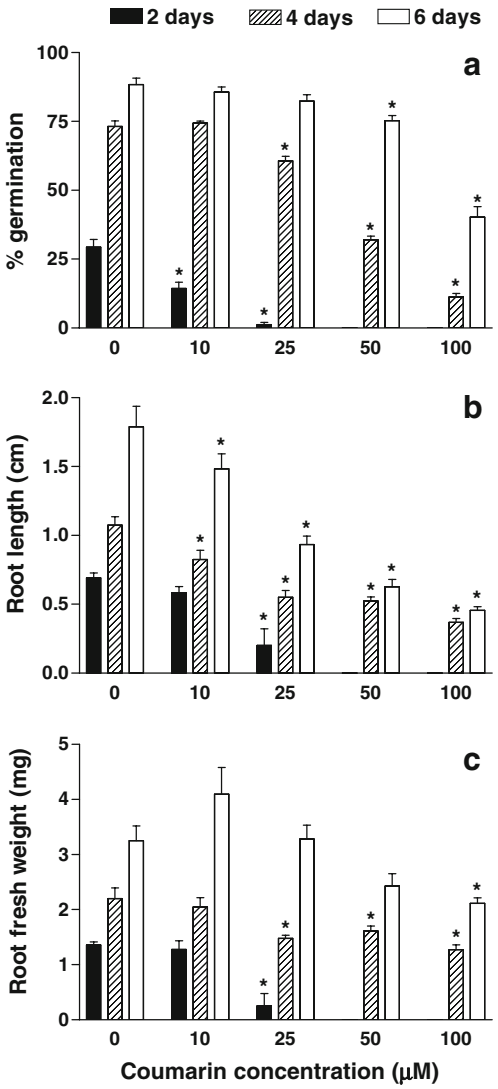

Fig. 1 The effects of coumarin on germination (**a**), root length (**b**), and root fresh weight (**c**) of *Bidens pilosa* L. Seeds were germinated and grown on the following regime: 8 hr light, at 30°C, 16 hr dark, at 20°C, photon flux density of approximately 230 μmol m^{-2} s^{-1}. Coumarin (10–100 μM) was added to the nutrient solution, and at each experimental interval (2, 4, or 6 d), roots were excised and their lengths and fresh weights were measured. All values are means of three to five independent experiments. *Error bars* are SEM. Significant differences between coumarin-treated and untreated seedlings were identified by ANOVA with Duncan's testing (*$P<0.05$)

28.4% on d 3 and 14.3% on d 4 relative to the value found on the second day.

The effects of coumarin at a concentration range of 10–100 μM were evaluated only in seedlings grown for 4 d (Fig. 3). At shorter time intervals, insufficient material was available for measurements because of the strong inhibition in seedling growth.

In contrast to what happened with germination and seedling growth (Fig. 1), overall respiration rates of root apices from seedlings grown for 4 d were stimulated in a dose-dependent manner by coumarin up to 50 μM

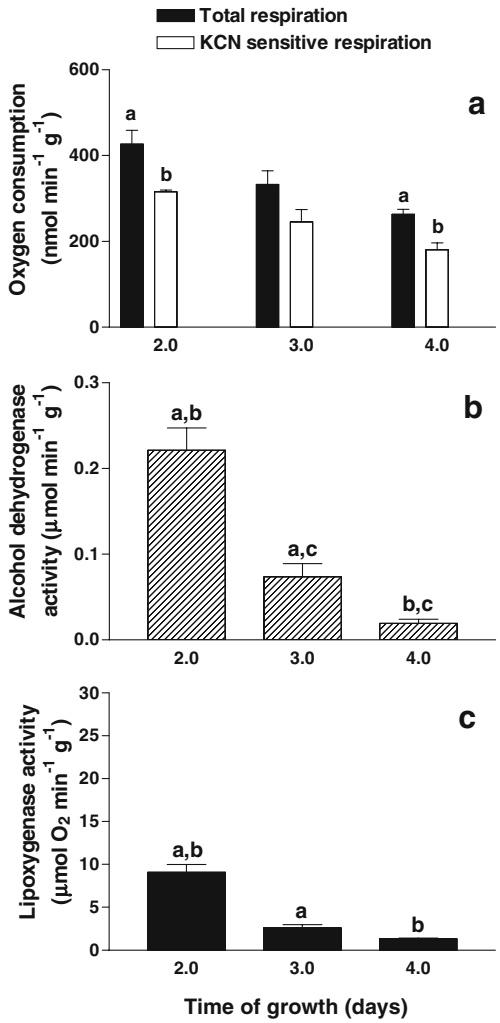

Fig. 2 Time course of respiration rates (**a**), alcohol dehydrogenase (**b**), and lipoxygenase (**c**) activities in roots of *B. pilosa* L. seedlings. In **a**, primary root tip samples were removed from seedlings and added without delay to the oxygen electrode vessel, containing 2.0 ml of nutrient medium in the absence or presence of 270 µM KCN. Oxygen consumption was followed polarographically over approximately 12–15 min. Total respiration: rate of oxygen consumption in the absence of inhibitors; KCN-sensitive respiration: difference between the rates of oxygen consumption measured in the absence and presence of KCN; Alcohol dehydrogenase activity (**b**) was measured in reaction medium containing 1.0 mM NAD^+ and 120 µM *n*-propanol. Lipoxygenase activity (**c**) was measured polarographically in the presence of 3.0 mM linolenic acid. Each data point is the mean value of four (**a**), three (**b**), or three (**c**) independent experiments. *Error bars* are SEM. *Pairs of letters* indicate statistical significance as determined by ANOVA with Duncan's testing ($P<0.05$)

(Fig. 3a). KCN-sensitive respiration was similarly stimulated, so that its relative contribution to overall respiration was not modified. It constituted 68.9% of overall respiration in the control condition and 67.8, 68.3, 70.8, and 66.4% in the presence of 10, 25, 50, and 100 µM coumarin, respectively.

Activities of alcohol dehydrogenase and lipoxygenase in root extracts from seedlings grown for 4 d were also stimulated in a dose-dependent manner by coumarin. The activity of alcohol dehydrogenase increased 4.6- and 9.3-fold, in the presence of 25 and 50 µM coumarin, respectively (Fig. 3b). Under the same conditions, the lipoxygenase activity increased 5.7- and 14.7-fold, compared to untreated seedlings (Fig. 3c).

To examine the possibility that lipoxygenase stimulation was a response to a cellular oxidative stress condition, we measured the content of malondialdehyde (MDA) and conjugated dienes in roots of seedlings grown in the presence of 25 and 50 µM coumarin (Table 3). An increment of nearly fourfold in conjugated dienes content

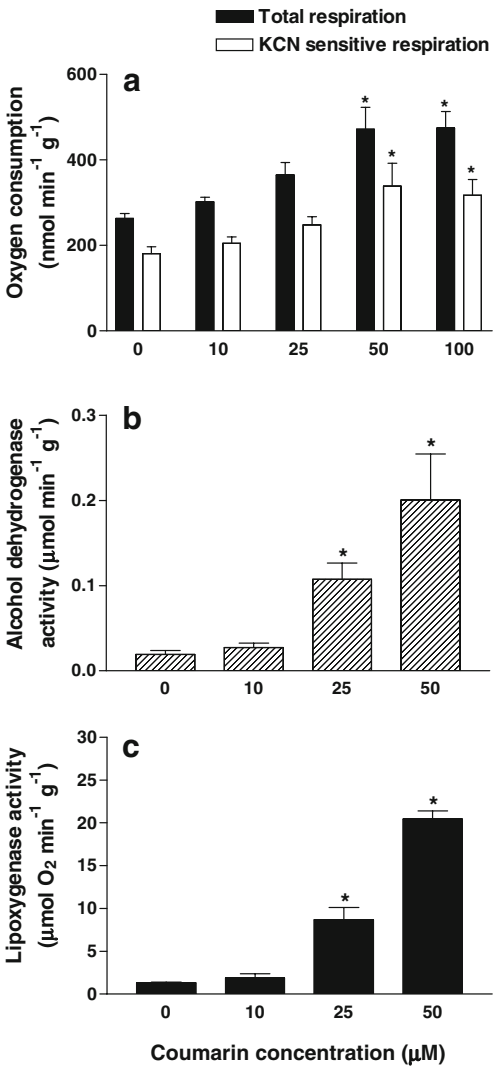

Fig. 3 Effects of coumarin on respiration rates (**a**), alcohol dehydrogenase (**b**), and lipoxygenase (**c**) activities in roots of *B. pilosa* L. seedlings. Seedlings were grown for 4 d in the absence or presence of coumarin (concentration range 10–100 µM). Respiration rates and the activities of lipoxygenase and alcohol dehydrogenase were measured as described in legend of Fig. 2. All values are the means of 4 (**a**), 3–4 (**b**), or 3 (**c**) independent experiments. *Error bars* are SEM. Significant differences between coumarin-treated and untreated seedlings were identified by ANOVA with Duncan's testing (*$P<0.05$)

Table 3 Effects of coumarin (25 and 50 μM) on the content of malondialdehyde (MDA) and conjugated dienes in primary roots from *Bidens pilosa* seedlings grown for 4 days

Coumarin Concentration	MDA (N=3)	Conjugated Dienes (N=6)
μM	nmol (g root fresh weight)$^{-1}$	μmol (g root fresh weight)$^{-1}$
0	14.3±2.4	3.18±0.21[a]
25	8.85±0.8	3.78±0.49[b]
50	12.3±1.4	15.68±2.06[a,b]

Values are expressed as mean±SE. Pairs of letters in each column indicate statistical significance as determined by ANOVA with Duncan's testing (P<0.05)

was found in 50 μM-treated seedlings, with no significant changes in MDA content.

Discussion

The study revealed different effects and potencies on *B. pilosa* for each compound tested. Coumarin was the most phytotoxic. Among the cinnamic derivatives, only ferulic and vanillic acids were active. Cinnamic acid derivatives possess in their structures a phenyl group with different substituents. Ferulic and vanillic acids possess in common the methoxyl substituent, which is absent in the other cinnamic acid derivatives. This group seems to be a critical factor in the inhibition of germination and growth of *B. pilosa*. This characteristic seems uncommon in other plant species such as *Arabidopsis thaliana* (Reigosa and Pazos-Malvido 2007) and *Amaranthus retroflexus* (Reigosa et al. 1999). In those studies, radicle growth of both species was reduced by ferulic and vanillic acids and also by *p*-coumaric and protocatechuic acids, which do not possess a methoxy group substituent.

Activities of the monoterpenes eucalyptol and camphor were also different. Both terpenes possess in common a hydrocarbonated cyclical structure and an oxygenated substituent, an ether function in eucalyptol and a ketone function in camphor. Camphor's higher solubility (Vaughn and Spencer 1993; Fischer et al. 1994) could have exerted some influence on its *B. pilosa* activity. Solubility did not seem to be a differential factor for coumarin and flavone activity. Both compounds are practically insoluble in water, but despite the fact that they are structurally related, flavone had weak activities on *B. pilosa*. The benzopyranone group of coumarin is an integral part of the structure of flavone, which possesses a phenyl substituent in position 2 of the benzopyranone group. This substituent apparently suppresses biological activity of the benzopyranone group in *B. pilosa*. This interpretation is consistent with the work of Richard et al. (1950), which demonstrated that the introduction of substituents in positions 3 and 4 of the benzopyranone group reduces the inhibitory potential of derivatives on the growth of *Avena* roots.

The phytotoxicity of coumarin seems to be species specific, as *E. heterophylla*, *S. obtusifolia*, and *I. grandifolia* were less sensitive. Coumarin and its derivatives are produced by plants of almost all families and are found on the surfaces of leaves, seeds, and fruits (Zobel and Brown 1995; Chon et al. 2003; Chon and Kim 2004; Khanh et al. 2006). There are a number of reports concerning the effects of coumarin on crop species, the responses also being species specific and concentration dependent (Murray et al. 1982). For example, at 680 μM, coumarin completely inhibits root growth of *Cucumis sativus* and *Zea mays* seedlings, but causes only slight inhibition of *Pisum sativum* root growth (Kupidlowska et al. 1994). Inhibition of *Triticum turgidum* ssp. *durum* seed germination was reported to occur at concentrations above 200 μM (Abenavoli et al. 2004, 2006). From this study, it is clear that *B. pilosa* has high sensitivity to coumarin in comparison with most assayed weed or crop species.

Several explanations for coumarin inhibitory action on germination have been proposed, including inhibition of cellulose synthesis (Hara et al. 1973), auxin-like activity (Jansson and Svensson 1980), inhibition of photosynthesis (Moreland and Novitzky 1987), uncoupling of mitochondrial oxidative phosphorylation (Knypl 1964; Yakushkina and Starikova 1978), blocking of the cell cycle (Zobel and Brown 1995), antimitotic action (Podbiekowska et al. 1994), inhibition of cell division and cell elongation (Svensson 1972), and inhibition of amino acid transport and protein synthesis (Van Sumere et al. 1972). The simplest explanation for the observed reduction in root seedling growth associated with increased KCN-sensitive respiration is that in *B. pilosa*, coumarin is acting as an uncoupler of mitochondrial oxidative phosphorylation. In this context, the observed increase in alcohol dehydrogenase activity would be interpreted as a compensatory increase of anaerobic ATP synthesis. However, comparisons between the metabolic parameters measured in seedlings grown in the presence of coumarin for 4 days and those of the control series (absence of coumarin) measured at days 2, 3, and 4 point to an alternative mechanism. Respiratory activity and the activities of alcohol dehydrogenase and lipoxygenase in the control series were higher shortly after the emergence of primary roots (d 2) and decreased progressively during the growth period. These results suggest that ATP production was provided by alcohol dehydrogenase activity only in the early stage of root growth with subsequent predominance of the mitochondrial ATP-generation pathway. The decline in KCN-sensitive respiration observed during the growth period

may represent a reduction in ATP demand due to progressive reduction in the root relative growth.

From these observations, it is plausible to suggest that the observed higher root respiration and alcohol dehydrogenase and lipoxygenase activities of seedlings grown for 4 days in the presence of coumarin are not consequences of an impairment on energy metabolism, but represent the metabolic status of seedlings at a different physiological age, i.e., in an earlier stage of growth. Coumarin probably acted by inducing a delay in seed germination and seedling growth. This conclusion is corroborated by the observation that the mean values of all parameters measured in seedlings grown in the presence of 50 μM coumarin for 4 days were statistically equal to those of seedlings grown for 2 days in the absence of coumarin. The only exception was the lipoxygenase activity, which was substantially higher in the presence of 50 μM coumarin.

Although the exact mechanism of germination and growth inhibition remains to be elucidated, our results corroborate the hypothesis that coumarin acts as a cytostatic agent, as suggested by its reported effects on mitosis and cell division (Svensson 1972; Podbiekowska et al. 1994; Zobel and Brown 1995).

Coumarin at higher concentrations also exerts an additional phytotoxic action in *B. pilosa* as indicated by the higher activity of lipoxygenase and the high content of conjugated dienes in root extracts. Activation of lipoxygenase is believed to be one of the immediate responses to changes in cell membrane structure induced by different agents including oxygen reactive species that can be generated in response to a variety of stress conditions (Siedow 1991; Porta and Rocha-Sosa 2002; Blokhina et al. 2003). The MDA content was presumably not increased because the products of lipid peroxidation were further oxidized or metabolized (Beuge and Aust 1978; Muscari et al. 1990). The hypothesis that coumarin induces a condition of oxidative stress was also suggested by Abenavoli et al. (2003, 2006) based on changes in antioxidant enzyme activities in durum wheat (*Triticum turgidum*) seedlings. In this plant species, however, the effects occurred at a concentration of 1,000 μM.

From the present study, it can be concluded that coumarin has a strong and selective ability to suppress the germination and growth of *B. pilosa* and might be effectively exploited as a natural herbicide. For example, the use of plants with high coumarin content (Macías et al. 1993; Chon et al. 2003; Chon and Kim 2004; Khanh et al. 2006) could be used in intercropping systems to reduce *B. pilosa* germination and growth.

Acknowledgements This work was supported by grants from the Fundação Araucária do Estado do Paraná and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Érica Marusa Pergo fellowship holder from the Conselho Nacional de Desenvolvimento Científico e Tecnológico. We are indebted to Dr. Adelar Bracht for suggestions on the revision of the present manuscript.

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