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Phytotoxicity Study on *Bidens sulphurea* Sch. Bip. as a Preliminary Approach for Weed Control

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Supporting Information

ABSTRACT: Farmers of the Franca region in Brazil observed that *Bidens sulphurea* was able to eliminate the *Panicum maximum* weed, which infected coffee plantations, without affecting the crop. In an effort to determine if the inhibitory effects observed were due to the presence of phytotoxic compounds from leaves and roots, a biodirected isolation and spectroscopic characterization has been carried out. The leaf dichloromethane and root acetone extracts were the most active, and the former appeared to be more phytotoxic to the target species, including four weeds. A total of 26 compounds were isolated from leaves and roots, and four of them are described here for the first time. The major compounds in the leaf extract are the sesquiterpene lactones costunolide, reynosin, and santamarine, and these showed marked inhibition. *Amaranthus viridis* and *Panicum maximum* were the most sensitive species of the weeds tested. These three phytotoxic lactones were also evaluated on *A. viridis* and *P. maximum* under hydroponic conditions. *A. viridis* was the most affected species with the three lactones, and santamarine was the most phytotoxic compound on both. This is the first time that the phytotoxicity of sesquiterpene lactones has been evaluated on hydroponic culture. The work described here is a preliminary approach for the use of *B. sulphurea* for weed control in agriculture, both as a cover crop and by use of its components as natural herbicide leads.

KEYWORDS: *allelopathy, Bidens sulphurea, costunolide, reynosin, santamarine, allelochemicals, hydroponic culture*

INTRODUCTION

For years, the farmers of the Franca region in Brazil have observed how the plant *Bidens sulphurea* was able to eliminate the *Panicum maximum* weed, which infected coffee plantations, without affecting the crop. They observed the absence of *P. maximum* in areas where *B. sulphurea* grows. For this reason, some farmers in the area use this plant as a weed management strategy by planting it in the line spacing of coffee crops.

The mechanism by which *B. sulphurea* inhibits weeds remains unknown, but this action could feasibly occur due to an allelopathic phenomenon.[1,2] Allelopathy is defined as the harmful or beneficial effects of a species of plant on the germination, growth, and/or development of other plants of the same or other species through the release of secondary chemical compounds, which are called allelochemicals, into the common environment.[3,4] *Bidens sulphurea* Sch. Bip. is a herbaceous plant that belongs to the family Asteraceae. This plant originates from North America and was introduced to Brazil as an ornamental plant.

The lack of crop rotation and excessive dependence on chemical control has favored the appearance of resistant and tolerant weeds. Cover crops represent a practice that has the potential to reduce the presence of problematic weeds. Such crops can be an important tool to reduce the population density of weeds using lower levels of herbicide without decreasing crop yield. Coverage methods provide benefits such as reducing pollution, erosion, insect pressure, fertilizer costs, and the use of herbicides and pesticides, reducing weeds, preserving moisture, and protecting water quality.[5−7]

Polyacetylenes, chalcones, phenylpropanoids, flavonols, thiophene derivatives, and arenes are among the allelochemicals most frequently found in species of the genus *Bidens*.[8−13] Extracts and fractions of several species of *Bidens* have been shown to display antiulcerogenic, antioxidant, anti-inflammatory, immunomodulatory, antihypertensive, antimicrobial, antiallergenic, antidiabetic, antiviral, antimalarial, and allelopathic activity.[14,15]

The aim of the study reported here was to determine whether the inhibition observed in the field with *B. sulphurea* could be due to the presence in the plant of compounds with phytotoxic activity. The first step was the evaluation of the phytotoxic activity of the plant in a preliminary bioassay in the laboratory. Active extracts and fractions were selected by a biodirected isolation.[16] The isolation, identification, and characterization of active compounds were subsequently carried out.

The bioactivities of fractions and compounds were evaluated on test plants (standard target species, STS)[17] (*Allium cepa* L., *Lactuca sativa* L., *Lepidium sativum*, and *Solanum lycopersicum* Mill.) and on weeds (*Amaranthus viridis* L., *Echinochloa crus-*

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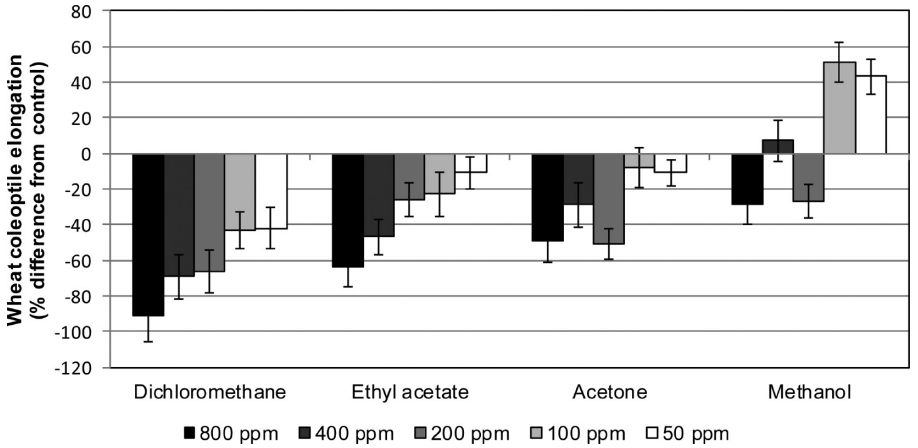

Figure 1. Effect of the five crude *B. sulphurea* leaf extracts (aqueous, dichloromethane, ethyl acetate, acetonic, and methanolic) on wheat coleoptile elongation. Values are expressed as percentage difference from control.

galli L., *Panicum maximum* Jacq., and *Urochloa decumbens* (Stapf) R. D. Webster). All of these weeds are problematic throughout the world.[18−21] Once the major phytotoxic compounds of the plant had been identified, they were evaluated on the hydroponic culture of the most sensitive species.

It is expected that the results will help to explain the behavior of *Bidens sulphurea* by demonstrating its potential for weed control in agriculture. This knowledge would allow the design of weed control strategies using this species. Furthermore, the discovery of new natural phytotoxins would provide new model compounds that could be used for the development of future natural herbicides.

MATERIALS AND METHODS

General Experimental Procedures. Infrared (IR) spectra (KBr) were recorded on a Fourier transform infrared (FT-IR) Spectrum 1000 spectrophotometer (PerkinElmer, Waltham, MA, USA). Nuclear magnetic resonance (NMR) spectra were acquired on 400, 500, and 600 MHz spectrometers (Agilent, Palo Alto, CA, USA). Chemical shifts are given in ppm with respect to residual ^{1}H signals of $CHCl_3$-d_1 (δ 7.25), and ^{13}C signals are referenced to the solvent signal (δ 77.00). Optical rotations were determined at room temperature on a model 241 polarimeter (PerkinElmer, Waltham, MA, USA) (on the sodium D line). HRMS were obtained on a Synapt G2 UPLC-QTOF ESI mass spectrometer (Waters, Milford, MA, USA). An ultrasonic bath (360 W, JP Selecta, Barcelona, Spain) was used for ultrasonic extraction.[22] Column chromatography was carried out on silica gel 0.060−0.200, 60A from Acros Organics (Geel, Belgium). Aluminum sheets coated with silica gel 60 F_{254} and 60 RP-18 F_{254s} (Merck, Darmstadt, Germany) were used for thin-layer chromatography. Compounds were viewed under $UV_{254/366}$ light after the plates were sprayed with H_2SO_4/H_2O/HOAc (4:16:80, v/v/v) followed by heating at 80 °C. High-performance liquid chromatography (HPLC; Merck-Hitachi, Tokyo, Japan) with refractive index detector was used (Elite LaChrom RI L-2490). Solvents were eluted by four-channel pumps (Elite LaChrom L-2130) with flow rates of 1 mL/min for analytical columns and 3 mL/min for semipreparative columns. The HPLC columns used were a semipreparative column (LiChrospher 10 μm 250-10 Si60, Merck) with a LiChrospher Si60 (Merck) guard column and an analytical column (Luna 5 μ, silica (2) 100 Å, 250 mm × 4.60 mm i.d., 5 μm, with Analytical Security Guard Cartridge System, Phenomenex, Torrance, CA, USA).

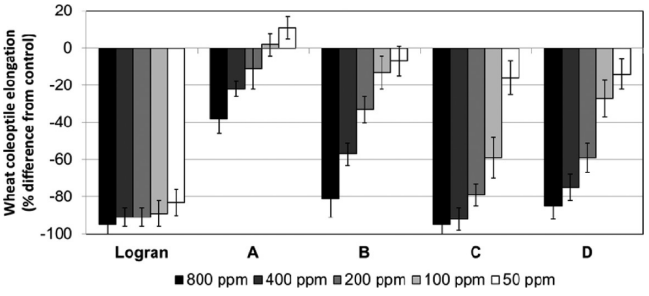

Figure 2. Effect of triasulfuron herbicide (Logran) and of the first four fractions obtained from the crude dichloromethane extract (A, B, C, and D) on wheat coleoptile elongation. Values are expressed as percentage difference from control.

Table 1. ^{1}H NMR (600 MHz) and ^{13}C (125 MHz) Spectroscopic Data for Compounds 23 and 24 in $CDCl_3$

	23		24	
no.	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		132.8		131.8
2	6.90 dd (8.0, 2.0)	118.5	6.82 dd (8.0, 2.0)	119.8
3	7.0 d (8.0)	122.8	6.88 d (8.0)	114.4
4		139.7		145.7
5		151.2		146.7
6	6.92 d (2.0)	110.2	6.89 d (2.0)	108.8
1′	4.15 d (4.5)	56.3	4.55 d (6.3)	74.5
2′	3.45 dt (7.0, 4.5)	56.0	3.87 m	74.4
3′a	4.07 dd (4.5, 12.5)	62.3	4.13 dd (3.6, 11.4)	65.1
3′b	3.85 dd (7.0, 12.5)		3.92 dd (5.9, 11.4)	
1″		175.2		177.5
2″	2.81 set (7.0)	33.9	2.58 set (7.0)	33.9
3″	1.31 d (7.0)	19.0	1.18 d (7.0)	19.0
4″	1.31 d (7.0)	19.0	1.17 d (7.0)	18.9
OCH_3	3.82 s	55.9	3.89 s	55.9

Organic Solvents. Acetone, ethyl acetate, chloroform, dichloromethane, methanol, and *n*-hexane for extraction, column chromatography, thin-layer chromatography, and HPLC were obtained from VWR International (Radnor, PA, USA). Deuterochloroform-d_1 MagniSolv with a deuteration degree minimum 99.95% for NMR spectroscopy was obtained from Merck (Darmstadt, Germany).

Plants. Leaves and roots were collected from *B. sulphurea* plants in full bloom in the city of Araraquara, São Paulo, Brazil (21°48′ to 21°50′ S and 48°10′ to 48°11′ W) during the rainy season (November 2013). The study material was deposited in the JABOTI Herbarium of the São Paulo State University (Universidade Estadual Paulista)/ School of Agrarian and Veterinary Sciences, Jaboticabal campus

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Table 2. ^{1}H NMR (600 MHz) and ^{13}C (125 MHz) Spectroscopic Data for Compounds 25, 26, and 20 in $CDCl_3$

	25		26		20	
no.	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		138.6		135.7		138.9
2	6.93 dd (7.6, 2.0)	118.7	6.92 dd (7.8, 2.0)	119.1	6.89 dd (7.7, 2.0)	118.9
3	6.98 d (7.6)	122.7	7.01 d (7.8)	122.9	6.98 d (7.7)	122.7
4		139.8		140.0		139.7
5		151.3		151.3		151.3
6	7.03 d (2.0)	110.5	6.94 d (2.0)	111.1	6.98 d (2.0)	110.6
1′	4.99 d (6.8)	73.5	5.81 d (6.4)	75.7	4.59 d (6.3)	74.1
2′	5.06 dt (6.3, 7.2)	77.7	3.94 m	74.6	3.88 m	74.0
3′a	3.81 dd (3.6, 11.4)	62.4	3.55 dd (3.6, 11.4)	62.7	4.14 dd (3.6, 11.4)	64.9
3′b	3.67 dd (5.9, 11.4)		3.47 dd (5.9, 11.4)		3.98 dd (5.9, 11.4)	
1″		175.2		175.1		175.3
2″	2.60 set (7.0)	34.1	2.65 set (7.0)	33.9	2.57 set (7.0)	33.9
3″	1.16 d (6.8)	18.9	1.21 d (6.8)	18.9	1.16 d (6.8)	18.9
4″	1.12 d (6.8)	18.9	1.18 d (6.8)	18.9	1.18 d (6.8)	18.9
1‴		177.2		176.5		177.5
2‴	2.82 set (7.0)	33.9	2.82 set (7.0)	34.1	2.81 set (7.0)	33.9
3‴	1.30 d (6.9)	19.0	1.31 d (6.9)	19.0	1.31 d (6.9)	19.0
4‴	1.30 d (6.9)	19.0	1.31 d (6.9)	19.0	1.31 d (6.9)	19.0
OCH_3	3.80 s	55.9	3.81 s	55.9	3.81 s	55.9

(voucher J. V. F. Martins et al., no. 01). Leaves and roots of *B. sulphurea* were left to dry in the shade and were then crushed in a Wiley mill (maximum particle size 1.5 mm), packed in paper bags, and stored in a cold, dry chamber.

Isolation of Compounds from Leaves. An initial extraction was performed using a small amount of the plant material in order to design and optimize the isolation process. For this purpose, 20 g of *B. sulphurea* dry leaf powder was initially extracted with hexane. The solvent hexane was used to remove oils, greases, and waxes (degreasing). The degreased material was extracted sequentially with organic solvents of increasing polarity: dichloromethane, ethyl acetate, acetone, methanol, and water. The ultrasound extraction technique was used in all extractions (three cycles of 15 min). At the end of the extraction period, each extract was filtered through 0.22-μm-pore paper in a Büchner funnel coupled to a vacuum pump and the solvent was then evaporated on a Rotavapor. All of the resulting crude extracts were evaluated in wheat coleoptile bioassays; the dichloromethane extract was found to be the most active (Figure 1).

A new extraction process was performed using a larger amount of plant material from the same source to obtain the crude dichloromethane extract, which was then fractionated and purified. To that end, the extraction technique described above was repeated, but in this case 480 g of *B. sulphurea* dry leaf powder was used. The plant material was first degreased with hexane, and the degreased material was then extracted with 2400 mL of dichloromethane. The extractions were performed in quadruplicate in 500 mL glass Erlenmeyer flasks containing approximately 100-g portions of plant material. Removal of the solvent provided the crude dichloromethane extract (DCM, 9.734 g).

The chlorophylls present in the DCM were not the substances of interest and could even interfere with the isolation process. As a consequence, it was decided to remove these compounds to facilitate the subsequent isolation and identification of bioactive substances. The chlorophylls were removed using a reverse-phase chromatographic column filled with RP-18 silica, eluting with mixtures of water and methanol at different polarities (0 to 100%) and finally with dichloromethane. Thin-layer chromatography (TLC) was used to identify possible similarities in the contents of the initial fractions, and four fractions of interest were obtained in addition to the final dichloromethane fraction from which the chlorophylls had been removed: fraction A (100% water), 258.6 mg; fraction B (20%, 40%, and 60% methanol), 1342.6 mg; fraction C (80% methanol), 1566.6 mg; fraction D (100% methanol), 2804.6 mg; chlorophyll fraction, 3386.5 mg. Fractions B, C, and D were the most active in coleoptile and seed bioassays (Figure 2).

Further fractionation of fractions B, C, and D was achieved using column chromatography (CC) with hexane/acetate mixtures of increasing polarity (0 to 100%) until all of the hexane had been replaced by ethyl acetate, which corresponded to the end of the elution. Upon completion of elution, the column was washed with 1.0 L of methanol. Throughout the chromatographic separation process, the fractions eluted by solvents of increasing polarity were analyzed by TLC. After separation by CC, fraction B yielded 12 subfractions (B1−B12), fraction C yielded 10 subfractions (C1−C10), and fraction D yielded 12 subfractions (D1−D12). The data of fractionation and isolation of compounds from leaves of the fractions B−D are presented in detail in the Supporting Information (Fractionation and Isolation from *B. sulphurea* leaves).

Compounds Isolated from Roots. An ultrasonic bath was used to extract compounds from the roots of *B. sulphurea* plants, adopting the same methodology as above. For this purpose, 150 g of dry root powder was extracted with acetone to yield 3.8 g of acetone extract after solvent removal. The root acetone extract was evaluated in the wheat coleoptile and seed bioassays and then fractionated by CC using a hexane/ethyl acetate gradient of 0 to 100% in 10% increments and finally with 100% methanol. Seven fractions (R1−R7) were obtained. The data of fractionation and isolation of compounds from roots of the fractions R2−R6 are presented in detail in the Supporting Information (Fractionation and Isolation from *B. sulphurea* roots).

Spectroscopic Data for the New Compounds. *1′,2′-Epoxy-4-hydroxy-3′-O-isobutyryl-Z-coniferyl alcohol (**23**):* colorless oil; $[\alpha]_D^{20}$ = −5.1° (*c* 0.2, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 275 (2.76), 239 (3.07) nm; IR ν_{max} (KBr) cm^{-1}; 3350 (OH); 1765, 1610 (PhOCOR); 1745 (−OCOR); 1510 ($PhOCH_3$). ^{1}H NMR ($CDCl_3$, 600 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1; positive-ion HREITOFMS *m*/*z* 266.1230 $[M + H]^+$ (calcd for $[M + H]^+$, 267.1232).

*1′,2′,4-Trihydroxy-3′-O-isobutyrylconiferyl alcohol (**24**):* colorless oil; $[\alpha]_D^{20}$ = +1.7° (*c* 0.2, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 278.9 (2.90), 242 (3.01) nm; IR ν_{max} (KBr) cm^{-1}; 3466 (OH); 1770, 1615 (PhOCOR); 1735 (−OCOR); 1520 ($PhOCH_3$). ^{1}H NMR ($CDCl_3$, 600 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1; positive-ion HREITOFMS *m*/*z* 283.1188 $[M + H]^+$ (calcd for $[M + H]^+$, 283.1182).

*1′,3′-Dihydroxy-2′,4-di-O-isobutyrylconiferyl alcohol (**25**):* colorless oil; $[\alpha]_D^{20}$ = +12.7° (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 275

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(2.80), 239 (2.77) nm; IR ν_{max} (KBr) cm^{-1}; 3468 (OH); 1767, 1610 (PhOCOR); 1740 (−OCOR); 1515 ($PhOCH_3$). ^1H NMR ($CDCl_3$, 600 MHz) data, see Table 2; ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 2; positive-ion HREITOFMS m/z 353.1596 $[M + H]^+$ (calcd for $[M + H]^+$, 353.1600).

*2′,3′-Dihydroxy-1′,4-di-O-isobutyrylconiferyl alcohol (**26**):* colorless oil; $[\alpha]_D^{20} = -7.8°$ (c 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 275 (2.80), 239 (2.79) nm; IR ν_{max} (KBr) cm^{-1}; 3350 (OH); 1770, 1615 (PhOCOR); 1735 (−OCOR); 1515 ($PhOCH_3$). ^1H NMR ($CDCl_3$, 600 MHz) data, see Table 2; ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 2; positive-ion HREITOFMS m/z 353.1592 $[M + H]^+$ (calcd for $[M + H]^+$, 353.1600).

Wheat Coleoptile Bioassay, Phytotoxicity Bioassay, and Hydroponic Bioassay. Wheat coleoptile, phytotoxicity, and hydroponic bioassays were performed according to procedures described in the Supporting Information.

RESULTS AND DISCUSSION

Chemical compounds involved in the inhibition of weeds observed in the field could come from the leaves and/or the roots. As a consequence, the extracts from both leaves and roots from *B. sulphurea* were studied. In the initial study, it was necessary to evaluate the phytotoxic activity of this plant, isolate and identify its compounds, and determine their activities. The leaves and roots were collected from Araraquara, São Paulo, Brazil, during the rainy season (November 2013).

Compounds and Bioactivities from *B. sulphurea* Leaves. Dried *B. sulphurea* leaves (60 g) were defatted with hexane and then sequentially extracted with dichloromethane (2.86% yield), ethyl acetate (0.87%), acetone (0.84%), and methanol (6.78%). The extracts were tested at concentrations of 800, 400, 200, 100, and 50 ppm in the coleoptile bioassay,[17] which is a rapid and sensitive test for a wide variety of bioactive substances.[23] The dichloromethane extract was the most active, and this was chosen for further study (Figure 1). Chlorophylls were removed from the defatted dichloromethane extract by using water/methanol mixtures of increasing polarity to afford four fractions, A (258.6 mg), B (1342.6 mg), C (1566.6 mg), and D (2804.6 mg), which were also evaluated in coleoptile bioassays at the concentrations described above. Fractions B, C, and D displayed the highest bioactivities (Figure 2). The phytotoxicity levels of these fractions were evaluated on STS and weed seeds at concentrations of 800, 400, 200, and 100 ppm (Figures S1 and S2).

All fractions showed inhibitory activity on the STS. The least affected seed was *L. sativa*, and fraction B was the only one that showed significant activity on germination of this species. Fractions B and C showed similar inhibitory activities on *L. sativum*, with inhibition of germination, root, and shoot length higher than 50% at the highest concentrations tested (800 ppm). Regarding *A. cepa*, shoot and root inhibition was about 70% at 800 ppm for fraction C. In *S. lycopersicum*, the inhibitory activity of the three fractions on the three parameters evaluated was approximately 50% at the highest concentration tested (Figure S1).

The three fractions also showed strong inhibitory activity on the weed species evaluated. Thus, fraction C showed the highest activity, with 60% or higher inhibition on root and shoot growth in the four studied weed species, excluding *E. crus-galli* shoots. *A. viridis* was the most markedly affected weed, with nearly 100% root and shoot growth inhibition at the highest concentrations (800 ppm) of the three fractions and at 400 ppm of fractions C and D (Figure S2).

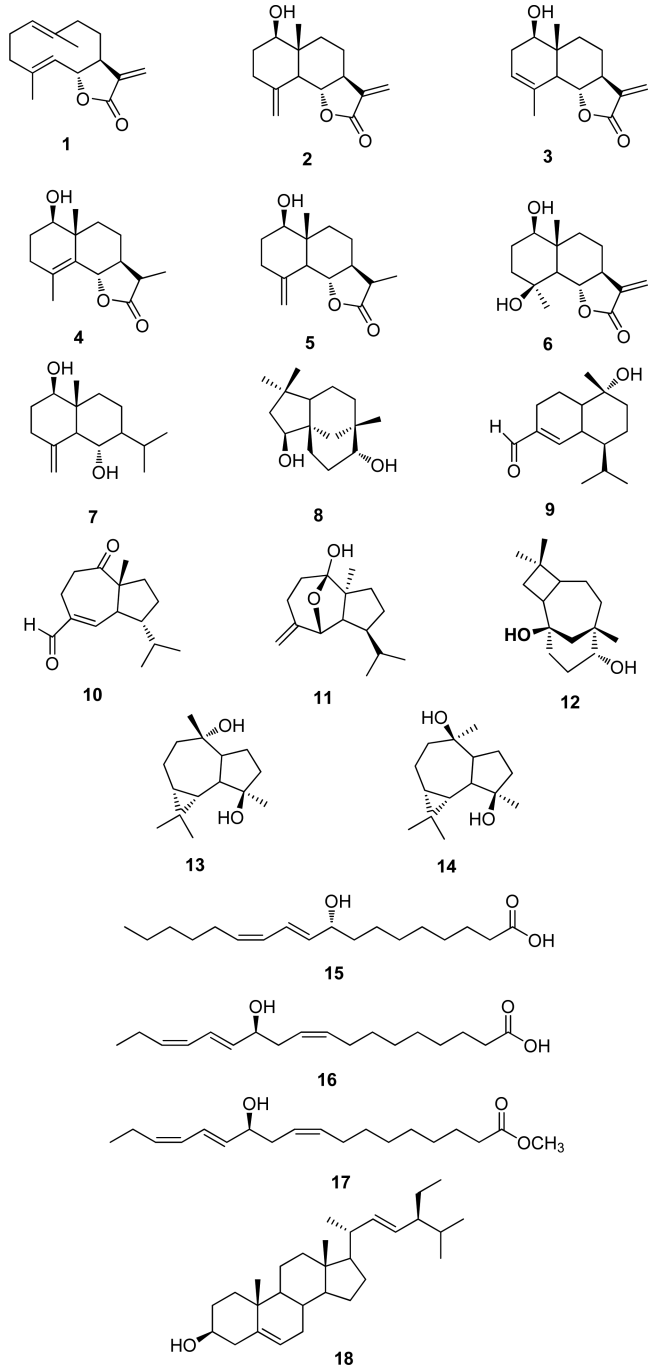

Figure 3. Compounds isolated from *B. sulphurea* leaves: costunolide (**1**), reynosin (**2**), santamarine (**3**), 11-epiartesin (**4**), 11α-13-dihydroreynosin (**5**), 1β,4β-dihydroxyarbusculin (**6**), 1β,6α-dihydroxyeudesm-4(5)-ene (**7**), clovane-2β-9α-diol (**8**), 10-hydroxy-15-oxo-α-cadinol (**9**), 10-oxoisodauc-3-en-15-al (**10**), 10-hydroxy-6,10-epoxy-4(14)-isodaucane (**11**), caryolane-1,9 β-diol (**12**), alloromadendrane-4β,10α-diol (**13**), alloromadendrane-4β,10β-diol (**14**), α-dimorphecolic acid (**15**), (9*Z*,12*S*,13*E*,15*Z*)-12-hydroxyoctadeca-9,13,15-trienoic acid (**16**), (9*Z*,12*S*,13*E*,15*Z*)-12-hydroxyoctadeca-9,13,15-trienoic acid methyl ester (**17**), and stigmasterol (**18**).

In short, it is concluded that the three fractions showed outstanding phytotoxic activity. Fraction C was the most active on both STS and weeds (Figures S1 and S2). The isolation and identification of the compounds present in fractions B, C, and D was therefore carried out in order to understand the

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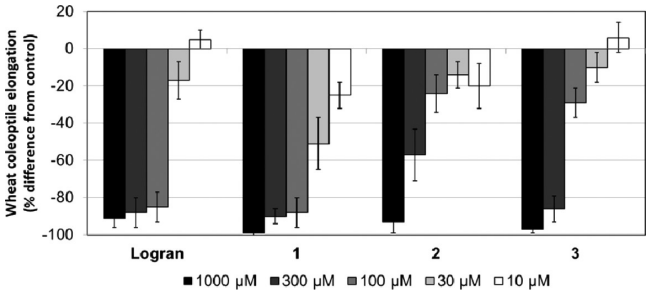

Figure 4. Effect of triasulfuron herbicide (Logran) and of the three major compounds obtained from *B. sulphurea* leaves (costunolide (**1**), reynosin (**2**), and santamarine (**3**) lactones) on wheat coleoptile elongation. Values are expressed as percentage difference from control.

agronomic behavior of *B. sulphurea* and to make it possible to use these compounds as natural herbicide models in the future. Compounds were purified using chromatographic techniques. The chromatographic separation and purification of leaf fractions B, C, and D resulted in the isolation of 18 compounds (Figure 3): 6 sesquiterpene lactones, 8 sesquiterpenes, 3 fatty acids, and 1 sterol. The compounds were identified by one- and two-dimensional NMR experiments, infrared (IR) spectroscopy, and mass spectrometry by comparison with the data reported in the literature as presented in the Supporting Information (Identified compounds from *B. sulphurea* leaves and roots).

Costunolide (**1**), reynosin (**2**), and santamarine (**3**) obtained from *B. sulphurea* leaves are characteristic compounds of

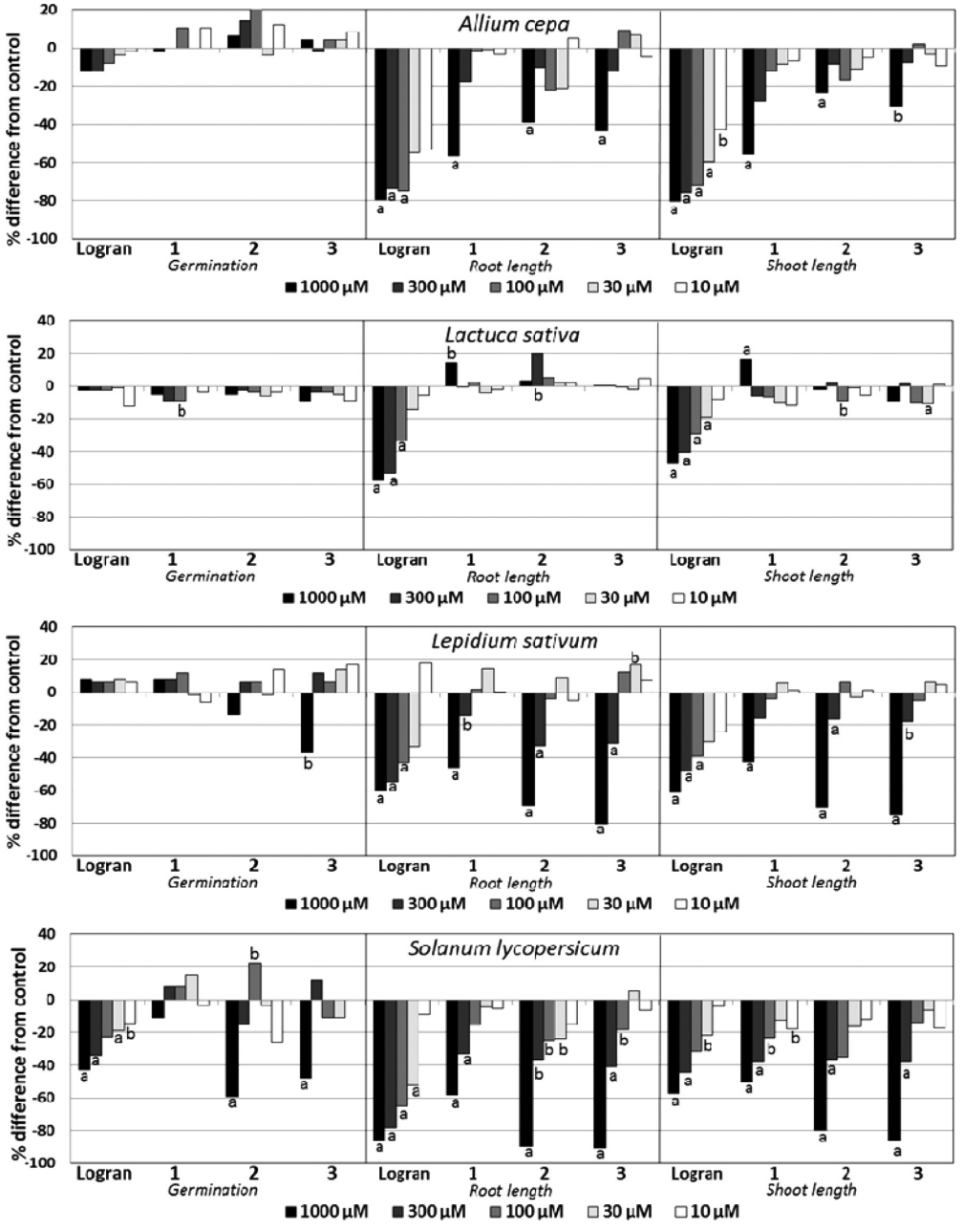

Figure 5. Effect of triasulfuron herbicide (Logran) and of the three major compounds obtained from *B. sulphurea* leaves (costunolide (**1**), reynosin (**2**), and santamarine (**3**) lactones) on the germination and growth of STS. Values are expressed as percentage difference from control. Significance levels $p < 0.01$ (a) or $0.01 < p < 0.05$ (b).

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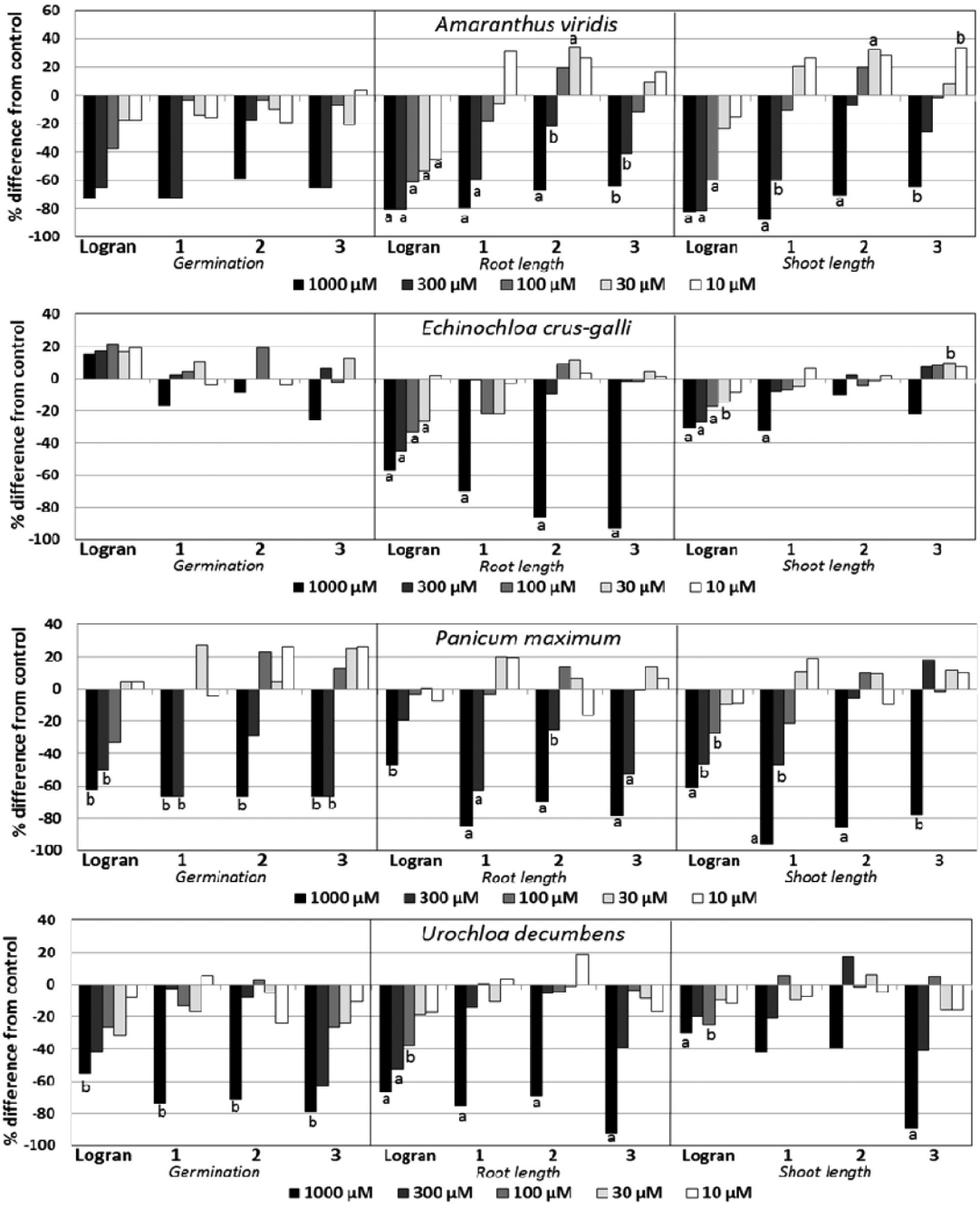

Figure 6. Effect of triasulfuron herbicide (Logran) and of the three major compounds obtained from *B. sulphurea* leaves (costunolide (**1**), reynosin (**2**), and santamarine (**3**) lactones) on weed germination and growth. Values are expressed as percentage difference from control. Significance levels $p < 0.01$ (a) or $0.01 < p < 0.05$ (b).

Asteraceae,[24] and these are the major sesquiterpene lactones. With the exception of costunolide, which has been isolated previously from this plant,[25] the other two characterized major compounds are described here for the first time in *B. sulphurea*.

The three major compounds were evaluated in a wheat coleoptile bioassay (Figure 4). Costunolide (**1**) demonstrated strong inhibitory activity on coleoptile growth, with greater than 90% inhibition at the three highest concentrations tested and more than 50% inhibition at 30 μM; these values were even higher than that achieved on using the herbicide Logran.

In general, the three major lactones tested were active at the highest concentration tested, 1000 μM, at which they showed 90 to 100% inhibition. The inhibitory activity decreased when lower concentrations were used, particularly in the case of reynosin (**2**), for which the activity was 57% at the second tested concentration (300 μM). The inhibition caused by santamarine (**3**) decreased less steeply with concentration, since it still caused over 80% inhibition at the second dilution. The results indicate that costunolide (**1**) is the most bioactive lactone, followed by santamarine (**3**) and reynosin (**2**) (Figure 4).

The coleoptile bioassay results allowed us to calculate the IC_{50} value for each product evaluated. A sigmoidal dose–response curve model was used to compare the IC_{50} values of the tested compounds. The IC_{50} values obtained were as follows: compound **1**, 24.1 μM mL^{-1} ($R^2 = 0.9921$); compound

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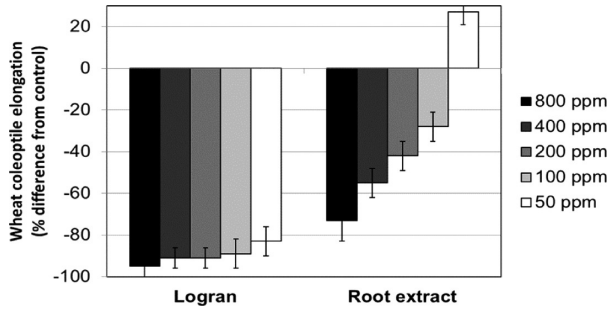

Figure 7. Effect of triasulfuron herbicide (Logran) and crude *B. sulphurea* root acetone extract on wheat coleoptile elongation. Values are expressed as percentage difference from control.

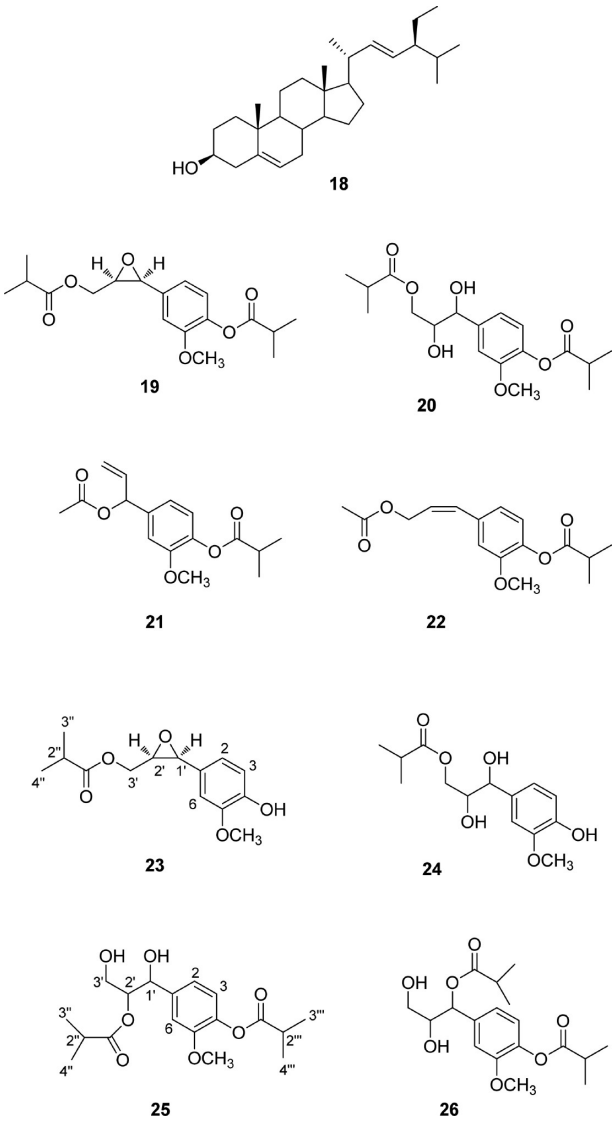

Figure 8. Compounds isolated from *B. sulphurea* roots: stigmasterol (**18**), 1′,2′-epoxy-3′,4-di-*O*-isobutyryl-*Z*-coniferyl alcohol (**19**), l′,2′-dihydroxy-3′,4-di-*O*-isobutyrylconiferyl alcohol (**20**), 1′-acetoxy-4-*O*-isobutyryleugenol (**21**), 3′-*O*-acetyl-4-*O*-isobutyryl-*Z*-coniferyl alcohol (**22**), 1′,2′-epoxy-4-hydroxy-3′-*O*-isobutyryl-*Z*-coniferyl alcohol (**23**), l′,2′,4-trihydroxy-3′-*O*-isobutyrylconiferyl alcohol (**24**), l′,3′-dihydroxy-2′,4-di-*O*-isobutyrylconiferyl alcohol (**25**), and 2′,3′-dihydroxy-1′,4-di-*O*-isobutyrylconiferyl alcohol (**26**).

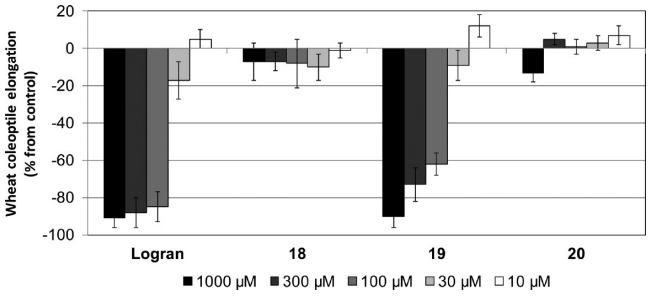

Figure 9. Effect of triasulfuron herbicide (Logran) and of the three major compounds obtained from *B. sulphurea* roots (stigmasterol (**18**), 1′,2′-epoxy-3′,4-di-*O*-isobutyryl-*Z*-coniferyl alcohol (**19**), and l′,2′-dihydroxy-3′,4-di-*O*-isobutyrylconiferyl alcohol (**20**)) on wheat coleoptile elongation. Values are expressed as percentage difference from control.

2, 285.3 μM mL^{-1} (R^2 = 0.9638); compound **3**, 139.8 μM mL^{-1} (R^2 = 0.9623); Logran, 39.5 μM mL^{-1} (R^2 = 0.9503). It was found that the IC_{50} values corroborated our previous conclusions regarding the relative activities of the products. The lowest IC_{50} value was that of costunolide (**1**), and this value is even lower than that of the commercial herbicide Logran.

The activity shown by the compounds correlated with that obtained for fractions in the coleoptile bioassay. Thus, the most active compound was costunolide (**1**), followed by santamarine (**3**) and reynosin (**2**). Fraction C, which was the most active, contained costunolide (**1**), reynosin (**2**), and santamarine (**3**) as its major compounds. Fraction D contained costunolide (**1**) at a lower concentration along with other minor components, and fraction B included reynosin (**2**) as its major component, as well as santamarine (**3**) and other minor components.

The compounds that showed the highest activities in the wheat coleoptile bioassay were also tested in a seed bioassay on STS (Figure 5) and four weed species (Figure 6). As in the results described above, all compounds exhibited high inhibitory activity, especially at the highest tested concentration (1000 μM). This inhibition decreased quickly with dilution (Figures 5 and 6).

Of the four evaluated STS species, *L. sativa* was the least affected, and this finding is consistent with the results of the bioassays performed with the fractions. The reynosin (**2**) and santamarine (**3**) lactones were the most active on *L. sativum* and *S. lycopersicum,* with inhibition values between 70% and 80% on roots and shoots at the first dilution of 1000 μM. The most active compound on *A. cepa* was costunolide (**1**), with root and shoot inhibition values close to 60% at the highest concentration tested (Figure 5).

The results on the four weed species also demonstrated the strong inhibitory activity of the three lactones. In most cases, the lactones were more active than the reference herbicide. Root growth was the most affected parameter in all tested seeds, with inhibition values between 60 and 90% at the highest tested concentration of all the evaluated compounds (Figure 6). The high sensitivity of the root to these compounds can be explained by the fact that the roots are the first part of the plant to emerge and to come into direct contact with allelochemicals, which are then absorbed directly. As a consequence, the roots are exposed to higher concentrations of allelopathic compounds than other parts of the plant.[22,26]

Shoots of *A. viridis* and *P. maximum* were also affected by all compounds, whereas *U. decumbens* shoots were only affected by

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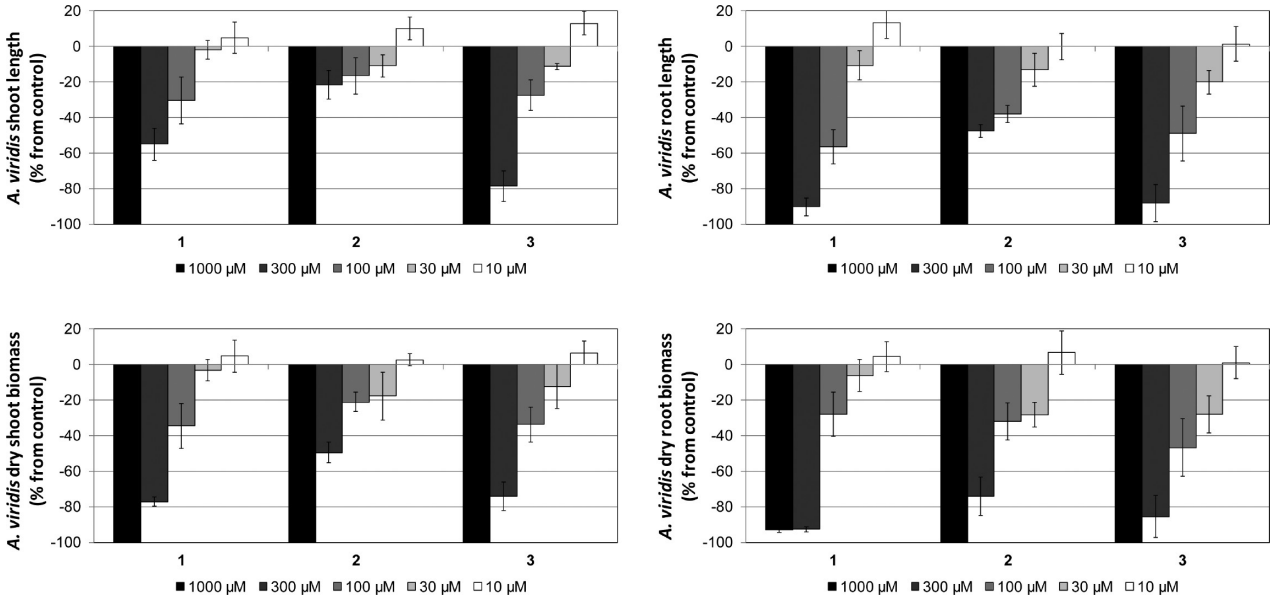

Figure 10. Effect of the three major compounds obtained from *B. sulphurea* leaves (costunolide (**1**), reynosin (**2**), and santamarine (**3**) lactones) on the hydroponic growth of shoot and root and dry shoot and root biomass on *A. viridis*. Values are expressed as percentage difference from control.

santamarine (**3**) and the growth of *E. crus-galli* shoots was not inhibited by any of the three compounds (Figure 6).

It can therefore be concluded that although all products inhibited the growth of the evaluated weeds, *A. viridis* and *P. maximum* were the most sensitive in terms of both the inhibitory levels achieved and the number of parameters affected. Furthermore, even at the second-highest concentration (300 μM), costunolide (**1**) and santamarine (**3**) maintained root growth inhibition levels of over 40% for these species, and the shoots were also inhibited by costunolide (**1**) (Figure 6).

Sesquiterpene lactones have diverse biological activities, including anti-inflammatory,[27] antibacterial,[27−29] anticancer,[30,31] and cytotoxic[32] activities. Studies have been conducted to evaluate the phytotoxic properties of sesquiterpene lactones.[22,33−36]

The results obtained in this study suggest that the inhibitory activity displayed by *B. sulphurea* leaf extract should be due to the presence of the three major sesquiterpene lactones isolated, which are biosynthesized in large quantities by *B. sulphurea*. Their phytotoxic activity suggests that they should be directly associated with the allelopathic potential presented by this species.

It is therefore concluded that the *B. sulphurea* leaf compounds have a phytotoxic effect and they can inhibit the growth of other plants. This ability can be explored for the use of this plant in weed control in agriculture. The major allelochemicals isolated from *B. sulphurea* leaves may offer a potential source of new structural types for model herbicides.

Compounds and Bioactivities from *B. sulphurea* Roots. As stated above, in order to gain an understanding of the behavior of this species, it is first necessary to carry out a phytochemical and phytotoxicity study of the complete plant. Once the phytochemical study of *B. sulphurea* leaves was completed, we proceeded to study the roots. Due to the lower quantity of root material available compared to leaves, extraction of the roots was only performed with acetone, a solvent that ensures the extraction of compounds with a wide range of polarities. The activity of the acetone extract was evaluated in the wheat coleoptile bioassay (Figure 7). The results showed that the root extract had inhibitory activity on coleoptile elongation and that the inhibitory profile of the extract decreased with dilution. The activities on STS (Figure S3) and weed seeds (Figure S4) were also evaluated. The root extract did not show phytotoxic activity on any of the evaluated species. Therefore, its components cannot be responsible for the allelopathic behavior of this species. In spite of this fact, the isolation and structural elucidation of compounds present in this extract were carried out in order to establish whether the leaf metabolites were also present, and to determine the compounds responsible for the activity shown by this extract in the coleoptile bioassay. An initial separation by column chromatography was performed using solvent mixtures of hexane and acetone with increasing polarity, which yielded seven fractions (R1−R7). Successive fractionations of five fractions R2−R6 obtained by column chromatography on silica gel followed by HPLC afforded compounds **18**−**26** (Figure 8): eight phenylpropanoids (**19**−**26**) and stigmasterol (**18**), which was also isolated from leaves. Compounds **19**−**22** have been described previously in the literature, and their spectroscopic data were identified by comparison as presented in the Supporting Information (Identified compounds from *B. sulphurea* leaves and roots). Four new phenylpropanoids were isolated (**23**−**26**) (Figure 8). Compound **23** was isolated as a colorless oil. The HRMS contained a peak at m/z 266.1230 $[M + H]^+$, and this is consistent with a molecular formula of $C_{14}H_{18}O_5$. The ^{1}H NMR spectrum of **23** exhibited common signals with those of **19**, with the absence of signals corresponding to the butanoyl moiety at the C-4 position of the aromatic ring, which should belong to a hydroxyl group (C-4 at δ 139.7) (Table 1). The *Z*-configuration of the epoxy structure was deduced from the coupling constant between H-1′ and H-2′ of 4.5 Hz.[37] The ^{1}H and ^{13}C NMR (mono- and two-dimensional spectra) data for compound **23** are consistent with the structure of an esterified *Z*-epoxy-coniferyl alcohol (Table 1).

The position of the OH moiety on the aromatic ring was determined with the aid of NOE measurements. Presaturation

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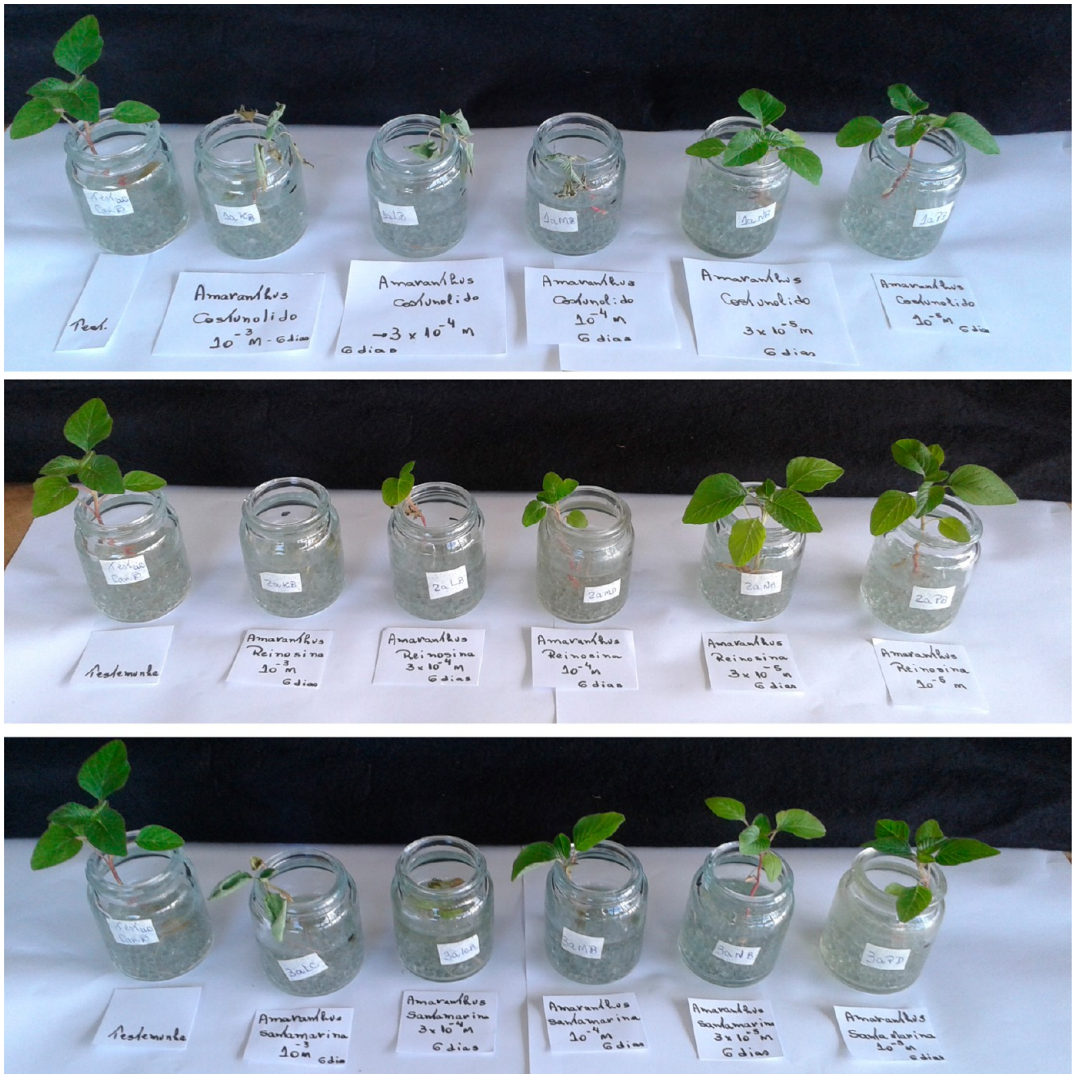

Figure 11. Effect of the three major compounds obtained from *B. sulphurea* leaves (costunolide (**1**), reynosin (**2**), and santamarine (**3**) lactones) on hydroponic growth with *A. viridis*.

of the methoxyl signal (δ 3.8) resulted in the enhancement of a signal of a meta-coupled proton (H-6) at δ 6.92 (Table 1). Thus, the structure of **23** was established as 1′,2′-epoxy-4-hydroxy-3′-*O*-isobutyryl-*Z*-coniferyl alcohol, which is described for the first time.

Compound **24** was isolated as a colorless oil. The positive ion mode HREITOFMS contained a peak at *m*/*z* 283.1188 $[M + H]^+$, which is consistent with a molecular formula of $C_{14}H_{20}O_6$. The ^{1}H NMR of **24** exhibited some similar signals to those of **20** with the absence of signals from a second butanoyl moiety, as occurred with compound **23** with respect to **19** (Figure 8) due to a hydroxyl group at C-4. The position of the OH group on the aromatic ring at C-4 was determined by NOE measurements, as in compound **23**. ^{1}H and ^{13}C NMR (mono- and two-dimensional spectra) data for compound **24** confirmed the structure 1′,2′,4-trihydroxy-3′-*O*-isobutyrylconiferyl alcohol for this new compound.

Compounds **25** and **26** were isolated as colorless oils, and both had the same molecular formula. Thus, the positive-ion HREITOFMS for compound **25** showed a peak at *m*/*z* 353.1596 $[M + H]^+$ (calcd for $[M + H]^+$, 353.1600) and *m*/*z* 353.1592 $[M + H]^+$ (calcd for $[M + H]^+$, 353.1600) for compound **26**, and both are consistent with the molecular formula $C_{18}H_{26}O_7$.

Furthermore, for compounds **25** and **26**, the ^{1}H NMR spectra are also in good agreement with the substitution pattern of the coniferyl alcohol (1-alkyl-4-acyloxy-5-methoxy substitution) found for compound **20**, with only differences in the shifts for H-1′, H-2′, and H-3′ signals for the three compounds. This fact can be explained by differences in the substitution pattern on the butanoyl group: at C-3′ for compound **20** (δ 4.14, H-3′a and δ 3.98, H-3′b), at C-2′ for compound **25** (δ 5.06, H-2′), and at C-1′ for compound **26** (δ 5.81, H-1′) (Table 2 and Figure 8). The mono- and two-dimensional ^{1}H NMR and ^{13}C NMR data are consistent with 1′,3′-dihydroxy-2′,4-di-*O*-isobutyrylconiferyl alcohol for **25** and 2′,3′-dihydroxy-1′,4-di-*O*-isobutyrylconiferyl alcohol for **26**; these are two new phenylpropanoids isolated from the *B. sulphurea* root.

The bioactivities of major phenypropanoids **19** and **20** as well as stigmasterol (**18**) were assessed in the wheat coleoptile bioassay in a concentration range from 1000 μM to 10 μM (Figure 9). The remaining compounds **21**−**26** were not tested due to the low amounts obtained. Compound **19** showed strong inhibitory activity on coleoptile elongation, whereas **18** and **20** were not active. The highest inhibitory activity of **19**

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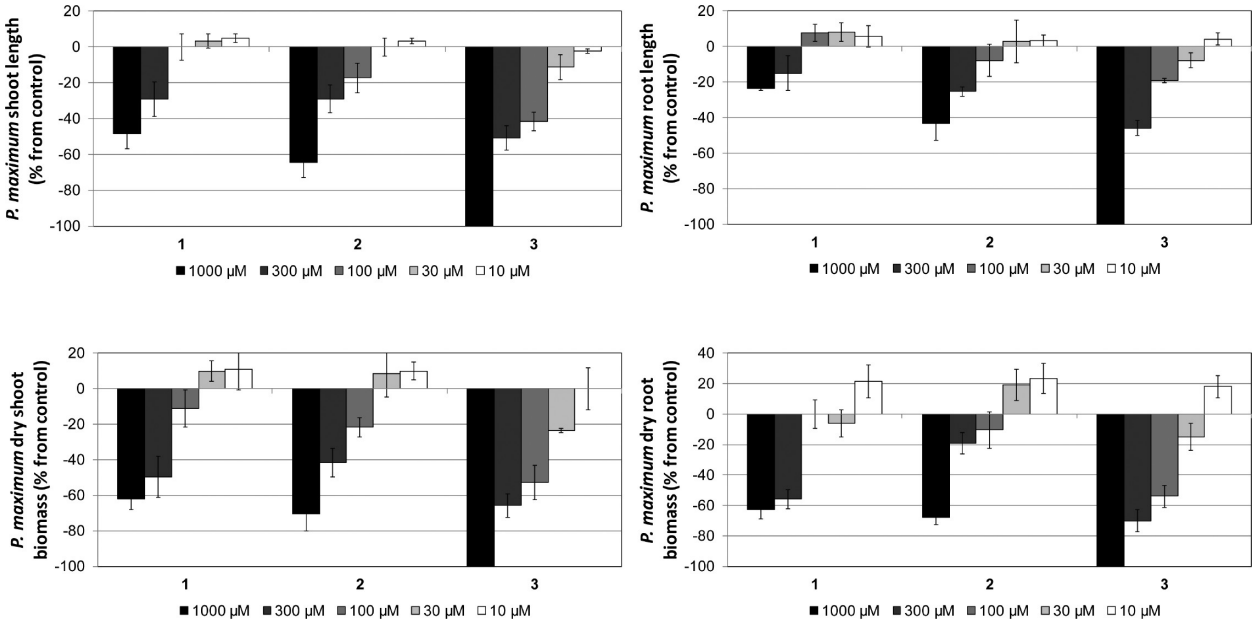

Figure 12. Effect of the three major compounds obtained from *B. sulphurea* leaves (costunolide (**1**), reynosin (**2**), and santamarine (**3**) lactones) on the hydroponic growth of shoot and root and dry shoot and root biomass on *P. maximum*. Values are expressed as percentage difference from control.

Figure 13. Effect of santamarine (**3**) obtained from *B. sulphurea* leaves on hydroponic growth with *P. maximum*.

showed a value of 90% at 1000 μM. This compound was also active at the second (300 μM, 73%) and the third (100 μM, 62%) concentrations, but almost no effect was observed at the highest dilutions. Some of these values were higher than the activity of the root extract (Figures 7 and 9). It is noteworthy that the only difference between these compounds is the presence of an epoxide (**19**) or a diol at the C-1′ and C-2′ positions (**20**) (Figure 8).

Phenylpropanoids have been reported to display antiulcerogenic,[38] antitumor,[39] and antifungal[38] activities. In the antifungal study the phenylpropanoids with an epoxy moiety showed activity against *C. albicans* versus nonactive compounds in which this ring was open, thus suggesting that this epoxy moiety is an important structural requirement for the activity of these compounds, as observed in our coleoptile bioassay with **19** and **20**.

The results obtained in this study suggest that the inhibitory activity displayed by *B. sulphurea* root extract on coleoptile elongation could be due to the presence of the major active phenylpropanoid **19**.

The leaf and root extracts from *B. sulphurea* have different chemical compositions. The three major active lactones extracted from the leaves were not found in the root extract, and the phenylpropanoids were not found in the leaf extract. The absence from the roots of the three active lactones responsible for leaf extract activity may explain why the root extract did not show phytotoxic activity against the tested seeds.

Hydroponic Bioassay. After the isolation and phytotoxicity study of the plant *B. sulphurea*, a bioassay was carried out under hydroponic conditions with the three major phytotoxic products from leaves, namely, costunolide (**1**), reynosin (**2**), and santamarine (**3**), on the two most affected weeds in the laboratory tests with these three sesquiterpene lactones, *A. viridis* and *P. maximum,* in order to evaluate their phytotoxic potential in the next level of bioassays. Compounds **1**, **2**, and **3** at the highest concentration (1000 μM) caused the death of *A. viridis* seedlings (Figure 10 and Figure 11). At the second concentration (300 μM) it was observed that costunolide (**1**) and santamarine (**3**) were the most phytotoxic compounds on *A. viridis*, with reductions in shoot length of 50% and 79%,

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respectively, and reductions of over 70% in dry biomass production of the aerial part. The root length of *A. viridis* was also considerably inhibited at a concentration of 300 μM, mainly by costunolide (**1**) and santamarine (**3**), both with reductions of 90% in relation to the values presented by the control plants. The dry biomass of the roots was also reduced by more than 80% by these two lactones (Figure 10). The length of roots was sensitive to the action of the two lactones, even at the third concentration (100 μM), with reductions of over 40%. When phytotoxic substances come into contact with the root they can directly affect the development and accumulation of dry mass as they interfere with cell division, membrane permeability, and enzyme activity.[40] Dilution of the lactones (30 μM and 10 μM) led to a decrease in the phytotoxic activity, and reductions in both shoot and roots from *A. viridis* did not exceed 20%.

In the case of *P. maximum*, it was observed that only the lactone santamarine (**3**) at the highest concentration (1000 μM) promoted the death of 100% of the seedlings (Figure 12 and Figure 13). At this concentration, reynosin (**2**) was the second most active lactone, followed by costunolide (**1**) (Figure 12). At 300 μM, the inhibitory levels reached by the lactones were reduced, but santamarine (**3**) maintained its phytotoxicity, and the accumulation of biomass both root and shoot was the most affected parameter, with average reductions of 70% (Figure 12). At 100 μM, santamarine (**3**) remained the most bioactive, with inhibitions on root and aerial part biomass of *P. maximum* of around 50%. Costunolide (**1**) and reynosin (**2**) at 30 μM did not show any significant activity on the growth parameters of *P. maximum*. Thus, considering the affected parameters and inhibition levels reached for *P. maximum*, santamarine (**3**) was the most phytotoxic lactone, followed by reynosin (**2**) and costunolide (**1**) (Figure 12). The results show that these compounds are phytotoxic to both weed species, *P. maximum* and *A. viridis*, under hydroponic conditions.

The results obtained in this study demonstrate that the inhibitory activity displayed by *B. sulphurea* leaf extract is due to the presence of the three major sesquiterpene lactones isolated. This is the first time that the phytotoxicity of sesquiterpene lactones has been evaluated in hydroponic culture, where *A. viridis* and *P. maximum* were affected. Costunolide (**1**), reynosin (**2**), and santamarine (**3**) are biosynthesized in large quantities by *B. sulphurea*, and, together with their activity on *P. maximum*, they are believed to be directly involved in the inhibitory behavior shown by this species on this weed in coffee plantations.

A further study of the presence of these compounds in soil is required to confirm the allelopathic activity of this species. This work represents a preliminary approach for the use of *B. sulphurea* for weed control in agriculture, both as a cover crop and by using its components as natural herbicide leads.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b01922.

Information on fractionation, isolation, and identification of compounds, the coleoptile, phytotoxicity, and hydroponic bioassays, and effects of triasulfuron herbicide (PDF)

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Notes

The authors declare no competing financial interest.

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