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Effects of sugarcane straw allelochemicals on growth and physiology of crops and weeds

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ABSTRACT

The phytotoxic activity of the compounds isolated was from sugarcane straw was evaluated on seedling growth of crops and weeds (*Brassica campestris, Lactuca sativa, Amaranthus quitensis, Bidens subalternans.* and *Sida rhombifolia*). Three compounds were identified : ferulic (FA), syringic (SA) and vanillic (VA) acids. VA drastically inhibited the root elongation, followed by FA and SA. These phytotoxins increased the root membrane permeability and depressed the root metabolic activity in *L. sativa*. VA and FA inhibited the mitotic index, while, SA increased the cell division. Vanillic acid inhibited the root branching, while, FA and SA stimulated the proliferation of secondary root. As the phenolic acids were often found concentrated in top soil layers under sugarcane straw, more studies are needed to establish their participation under field conditions.

Key word: Bioassay-guided isolation, phenolic acids, physiological effects, sugarcane straw leachate.

INTRODUCTION

Argentina sugarcane (*Saccharum officinarum*) straw is left in the field after harvest to prevent soil erosion and environmental pollution from its burning (15). Straw retention on soil surface also suppresses the growth of weeds, reducing the use of herbicides (12). In greenhouse experiments, sugarcane straw leachates inhibits the weeds growth (11). Although some compounds with biological activities have been isolated from sugarcane straw (9,13) but phytotoxins were rarely identified. Recently, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2-benzoxazolinone (BOA) were isolated from sugarcane leaves (16). It is possible that some unknown phytotoxins remains to be isolated from sugarcane straw. The purpose of this study was: (i) to isolate and identify the phytotoxic compounds of sugarcane straw leachate, (ii) to determine the relative phytotoxic activity of isolated compounds on weeds and on physiological processes of *Lactuca sativa* and (iii) to determine the concentration of isolated compounds in sugarcane straw leachate.

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MATERIALS AND METHODS

Plant material: *Saccharum officinarum* L. var Tuc (CP) 77-42 straw was collected from Las Talitas (Tucuman, Argentina). Then, straw was dried at 60 °C for 48 h in oven. Seeds of *Brassica campestris* L. *Lactuca sativa* L. cv Grand Rapids, *Amaranthus quitensis* L., *Bidens subalternans* L. and *Sida rhombifolia* L. were sterilized with 1% sodium hypochlorite for 30 min, rinsed with sterile distilled water, dried with sterile filter paper and germinated in water. The germinated seeds with root length of 1 mm were used in bioassays.

Straw leachate preparation: Dry straw (70 g) was soaked in 1.0 l sterile distilled water and shaken for 4 h at 25 °C dark. The leachate was vacuum filtered through a filter paper (Whatman #1) and centrifuged at 23428 g for 15 min at 4 °C. The supernatants were stored at –20 °C until use. Leachate dilutions were prepared with sterile distilled water and filtered through 0.22 μm nylon filter membranes (Millipore).

Chromatography: Column chromatography (CC) was performed on Sephadex LH 20 (800 x 27 mm, Amersham Pharmacia Biotech) and Silica gel 60 (0.2–0.5 mm, 240 x 16 mm,Merck). HPLC operations utilized IB-SIL RP 18 (5 μm, 250x 10 mm and 4.6 mm, Phenomenex) columns and a Gilson 118 UV detector. GC–MS analysis were carried out on a GC-HP 6890 with a quadrupole mass selective detector HP 5973, source 70 eV, fitted with a HP-5 MS column (5% phenylmethylsiloxane, 30 m, 0.25 mm id, 0.25 μm film thickness) using helium as carrier gas (1.0 mL min^{-1}). UV–Vis absorption spectra were recorded (200-400 nm) using a spectrophotometer Beckman DU-650. Known compounds were identified by comparison of HPLC retention times, MS and UV-Vis absorption spectra with literature data and analytical grade standards. The identified compounds were quantified by HPLC using external standards.

Isolation and identification of phytotoxic compounds: The leachate (70 g of dry straw l^{-1}) was lyophilized to dryness. The residue (776.8 mg) was dissolved in 50 mL water and successively extracted with diethyl ether followed by ethyl acetate. The organic fractions were dried in Rotary Evaporator at 50 °C, dissolved in 3 mL methanol (MeOH) and clarified by centrifugation.

The diethyl ether fraction (8.74 mg) was fractionated by gel filtration CC using MeOH as eluent. Fractions were monitored at 280, 310 and 350 nm. Five fractions were separated (L1 – L5). Fraction L3 (4.85 mg) was loaded on a Silica gel 60 column and eluted with chloroform – acetic acid (100 mL 90:10; 100 mL 80:20; 50 mL 70:30 and 50 mL 50:50, v/v). Six fractions (S1-S6) were obtained and concentrated under reduced pressure.

Fraction S1 (0.04 mg) was injected via a Rheodyne valve with a 500 µl loop into a micropreparative HPLC column. The elution was performed at a flow rate of 2 mL min^{-1} with solvent A (2% acetic acid in water) and B (2% acetic acid in MeOH) in the ratio of 35 % B for 5 min, increasing to 90 % B in 15 min; after 5 min at 90% B, the column was

cleaned-up decreasing to 35% B in 5 min. Re-equilibration was done at 35% B for 20 min. Compounds were detected at a wavelength of 266 nm. The retention times (min) were 24.77 (**1**), 28.38 (**2**), 31.28 (**3**) and 32.27 (**4**). The detected peaks were collected and analyzed by TLC and analytical HPLC. The UV-Vis absorption spectra of each isolated compound were recorded. The compounds and standards were permethylated with diazomethane before injection to GC- MS according to Fieser and Fieser (7). GC-MS conditions were as follows: 2 μl of the sample were injected to the capillary column and the temperature gradient was: from 90 to 270°C, 90 °C (2 min), 90 to 150°C (3 min), 150 °C (1 min), 150 to 270°C (9 min) and 270°C (1 min).

Leachate content of isolated phytochemicals: The isolated compounds were quantified in the leachate using external standard method by HPLC. Calibration curves were built with authentic vanillic (VA), ferulic (FA) and syringic (SA) acids (> 99%, Aldrich). A gradient elution was performed to separate phenolic acids in the leachate at a flow rate of 1 mL min^{-1} with solvent A (2% acetic acid in water) and B (2% acetic acid in MeOH) as follow: 0% B to 30% B in 30 min; 30% B to 42% B in 12 min; 42% B to 100% B in 4 min. Compounds were detected at 266 nm.

Phytochemical screening: The components of the straw leachate and column fractions were separated on silica gel 60 F_{254} plates (0.25 mm layer, Merck). Ethyl acetate – formic acid – acetic acid - water (100:11:11:26, v/v) and benzene - dioxane – acetic acid (90:25:4, v/v) were used as development solvents. The separated components were visualized under UV light (254 and 366 nm, UV Lamp Model UV 5L-58 Mineralight Lamp) and sprayed with 1% ferric chloride in MeOH.

Determination of total phenolics: Total phenolic compounds were estimated using the method of Singleton *et al.* (17) with Folin-Ciocalteau reagent. Ferulic acid was used as standard.

pH: Aqueous solutions and water controls assayed were buffered with 5 mM 2-[*N*-morpholino] ethanosulfonic acid (MES) adjusted to pH 5.7 with 1 N sodium hydroxide.

Bioassay in 24-well plates: The biological activity of column fractions was assayed as previously reported for *L. sativa* in 24-well plates (14). The amounts of fractions assayed were 0.02, 0.10, 0.20, 0.30, 0.40 and 0.50 µmol of total phenolic compounds per well in 200 µl of 5 mM MES (pH 5.7). Mannitol solutions prepared in MES (pH 5.7) with the same osmotic potential as the assayed samples were also assayed. Five *L. sativa* seeds were placed on filter paper (Whatman #1) set at the bottom of each well. The plates were sealed with parafilm and placed in the growth chamber at 25 ± 1 °C with a 12 h photoperiod at 400 μmol m^{-2} s^{-1} photosynthetically active radiation. Root length of *L. sativa* was measured in 5 d-old-seedlings. Each treatment consisted of 4 replications and was repeated twice. HPLC analysis confirmed the isomerization of FA in the assayed growth conditions in the ratio 2:1 (*trans*: *cis*).

Bioassay in Petri dishes: Aqueous solutions of vanillic, ferulic and syringic acids with concentrations between 0.1 and 4.0 mM and leachate dilutions with concentrations of total

phenolic compounds between 0.1 and 4.0 mM were assayed in 6 cm Petri dishes. Mannitol solutions with the same osmotic pressure as the dilutions of straw leachate (25-75 mmol kg^{-1}, SemiMikro osmometer, Knauer) and distilled water were also assayed. In each Petri dish, 17 germinated seeds of a test plant were placed on a layer of filter paper and watered with 3 mL of a test solution. The dishes were sealed with parafilm and placed in a growth chamber outlined previously. After 5 d root and shoot length were measured.

Changes in root morphology: A set of *L. sativa* seedlings were grown in solutions of phenolic acids with concentrations of 0.1 to 1.0 mM in the Petri dish bioassay previously indicated. After 5 d the seedlings were transplanted to unsterile soil and grown in the growth chamber for 30 d. Then, the elongation of the primary root seedlings and the number of secondary roots were determined. The experiment was performed in triplicate and twice-repeated.

Leakage test: Changes in membrane permeability due to the identified phenolic acids were assayed by measuring leakage of solutes and ultraviolet absorbing materials. Samples of excised radicles (0.3 g fresh weight) from 5 day-old *L. sativa* seedlings were suspended in 20 mL aqueous solutions (pH 5.7) of FA, SA and VA at concentrations between 0.1 and 1 mM after 48 h at 25 °C. After three washings in distilled water, roots were suspended in 15 mL deionized water for 4 h at the same temperature. Then, electric conductivity was analyzed (DDS – 11A). The UV-absorbing materials (e.g. amino acids, nucleotides, polypeptides, etc) were determined by measuring the absorbance at 260 nm. The experiment was performed in triplicate and twice-repeated.

Root reduction activity test: *L. sativa* roots (100 mg of fresh weight) grown in the phenolic acids solutions (pH 5.7) were washed, blot-dried, weighed and soaked in 5 mL of 2 g L^{-1} of 2,3,5-triphenyl tetrazolium chloride (TTC) dissolved in a 50 mM PO_4 buffer (pH 7) at 37 °C for 4 h in the dark. Then, 0.5 mL of 1 M sulfuric acid was added to stop the reaction. Roots were removed, washed with distilled water, blot-dried and ground with two portions of 3 to 4 mL ethyl acetate in a mortar and pestle. The extract was filtered through filter paper. The volume was made up to 7 mL and the optical density of the formazan production was read at 485 nm. The experiment was performed in triplicate and twice-repeated.

Mitotic index test: Inhibition of *L. sativa* root cell division (mitotic index) was measured according to Armbruster *et al.* (2) with some modifications. A filter paper (Whatman #1) and 17 surface sterilized L. *sativa* seeds were placed in 6-cm Petri dishes. Three mL of aqueous solutions of ferulic, vanillic and syringic acids were added in each Petri dish. The phenolic acids were assayed at the concentrations of 0.1, 0.5 and 1 mM (pH 5.7). Dishes were sealed with Parafilm and placed in the growth chamber outlined previously. After 6 days, root tips were fixed in glacial acetic acid / absolute ethanol (1:3) for 24 h and stored in 70% ethanol at 5 °C until use. Root tips were then hydrolyzed in 2 N chloride acid for 15 min at 60 °C and then repeatedly washed with distilled water. Root tips were stained in the dark for 15 min using Schiff's reagent (8) and then squashed in 15 g L^{-1} carmine dissolved in 45% glacial acetic acid. Mitotic stages were observed in at least 1000 cells per slide at a magnification of 40x with a Olympus CH microscope.

Statistical analysis: Data in tables and figures were presented as means ± standard error. Data of measured physiological processes previously indicated were subjected to analysis of variance (ANOVA) with significant differences between the means identified by GLM procedures. The results were given in the text as probability values, with $P < 0.05$ adopted as the criterion of significance. Differences between treatment means were established with Dunnet T3. Non-linear regression procedures were needed to analyze root elongation response against phenolic acid concentrations. Complete statistical analysis was performed with SPSS 7.5 for Windows.

RESULTS AND DISCUSSION

The components extracted in diethyl ether fraction inhibited the root elongation of *L. sativa* more than those extracted by ethyl acetate. Separation of diethyl ether components by a Sephadex LH 20 yielded five peaks (L1 to L5, Figure 1). L3 peak was most active on the plant growth. The constituents of L3 were loaded on a silica gel column and separated in six fractions (S1 to S6). S1 was the most phytotoxic and its constituents were separated in four peaks (1,2,3 and 4), by micropreparative HPLC. Each peak comprised a pure compound. The UV-Vis and MS spectra of these compounds indicated that they were hydroxybenzoic acids (vanillic and syringic acids) and isomers of a hydroxycinnamic acid (*trans*-ferulic and *cis*-ferulic acids) (Table 1). These compounds are commonly present in angiosperms (19). Furthermore, plant residues localized on soil surface are often the main source of these phytotoxins in managed ecosystems (10).

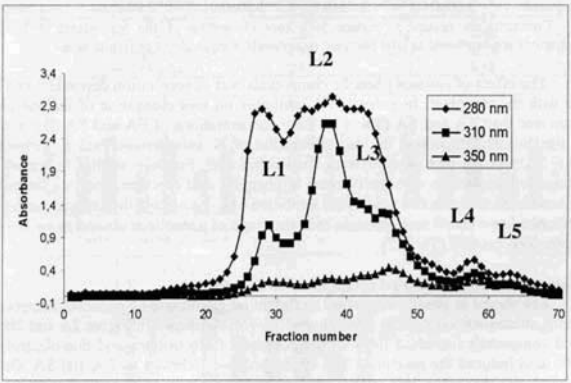

Figure 1. Elution profile on Sephadex LH 20 of the ether fraction extracted from sugarcane (*Saccharum officinarum* L.) straw.

Effect of isolated compounds on seedling growth

Only root elongation was shown because leachate dilutions and their isolated constituents did not affect shoot growth of test plants (Table 2). Mannitol solutions with the same osmotic potential and pH as the leachate dilutions had no effect on seedling growth of test plants (not shown) suggesting that leachate organic constituents were responsible for the observed growth inhibition.

Table 1. Absorption maxima and retention times of isolated compounds

Compound isolated	Absorption maxima (UV-Vis spectra)	Retention time (micropreparative HPLC)
Vanillic acid (**I**)	208, 255 y 288 nm	24,77 min
Syringic acid (**II**)	212 y 262 nm	28,38 min
cis-Ferulic acid (**III**)	217, 233, 321 y 324 nm	31,28 min
trans-Ferulic acid (**IV**)	217, 233, 321 y 324 nm	32,27 min

Table 2. Effects sugarcane straw leachate dilutions and their identified phytochemicals on radicle elongation of weeds and *Lactuca sativa* L. Data are shown as mean ± SE.

Treatment EC_{50} (mM)	Test plant spp.				
	Brassica campestris	*Lactuca sativa*	*Amaranthus quitensis*	*Bidens subalternans*	*Sida rhombifolia*
Straw leachate[b]	0.43±0.04	0.42±0.03	0.80±0.01	0.97±0.02	0.88±0.03
Vanillic acid	0.40±0.02	0.45±0.01	0.80±0.01	1.80±0.04	0.70±0.03
Ferulic acid	1.90±0.04	1.20±0.03	1.25±0.01	2.00±0.02	2.00±0.01
Syringic acid	3.60±0.02	2.75±0.05	3.50±0.01	2.90±0.02	1.50±0.02

EC_{50} – Concentration needed to reduce 50% root elongation of the test plants, *b* Leachate concentrations are expressed as total phenolic compounds in equivalents of ferulic acid.

The effect of isolated phenolic compounds was concentration dependent and also varied with the test plant. In general, VA inhibition on root elongation of the test plants was stronger than FA and SA (Table 2). Low concentrations of FA and SA (0.1 and 0.5 mM) significantly stimulated the root elongation of *B. subalternans* and *L. sativa* (not shown). SA was the less phytotoxic isolated compound. Previous studies indicated that root length could decline with the increase in phenolic acid concentration in a linear or a curvilinear fashion (5). In this study, root inhibition of *L. sativa* and the assayed weeds was best described as a curvilinear response over the range of phenolic acids and straw leachate concentrations assayed (Table 3).

Physiological effects of isolated compounds on *L. sativa*

Membrane is usually suggested as the initial action site of phenolic compounds, triggering subsequent changes in plant physiology (6). As shown in Figure 2A and 2B, the isolated compounds increased the root cell leakage of electrolytes and non-electrolytes. Vanillic acid induced the maximum loss of electrolytes, followed by FA and SA. On the other hand, the maximum loss of non electrolytes was observed in lettuce roots treated with FA, followed by VA and SA. The efflux of organic and inorganic cell constituents indicated that phenolic acids damaged the root membrane integrity (3).

Lettuce roots grown in the control (MES buffer solution) produced more formazan than those grown in solutions of phenolic acids. When compared with VA and FA, roots treated with SA significantly produced more formazan (Figure 1C). Formazan production indicates the mitochondrial dehydrogenase activity (18). The decrease in dehydrogenase activity may associate with decrease in ATP production. Low ATP production may induce the inhibition of root growth.

Table 3. Partial regression coefficients and R^2 values for root elongation (% of control) of test plants in presence of phenolic acids (ferulic, vanillic and syringic) and straw leachate

Test plant	Phenolic acid	Line intercept	Linear	Quadratic	R^2 (Quadratic regression)
Amaranthus quitensis	Vanillic Acid (VA)	2.15	-1.67	0.51	0.92
	Ferulic Acid (FA)	2.24	-1.31	0.35	0.96
	Syringic Acid (SA)	2.15	-0.88	0.30	0.88
	Straw leachate (SL)	2.02	-1.44	0.41	0.81
Bidens subalternans	Vanillic Acid	1.53	-0.81	0.24	0.92
	Ferulic Acid	1.64	1.21	-0.55	0.84
	Syringic Acid	1.84	0.64	-0.24	0.81
	Straw leachate	2.02	-1.44	0.41	0.98
Brassica campestris	Vanillic Acid	2.82	-2.72	0.85	0.89
	Ferulic Acid	3.17	-1.48	0.61	0.91
	Syringic Acid	3.15	0.26	-0.50	0.94
	Straw leachate	2.91	-3.10	0.95	0.95
Lactuca sativa	Vanillic Acid	2.34	-2.76	0.93	0.94
	Ferulic Acid	2.77	-1.57	0.24	0.85
	Syringic Acid	2.61	0.91	-0.54	0.79
	Straw leachate	2.15	-2.38	0.78	0.98
Sida rhombifolia	Vanillic Acid	2.34	-1.86	0.53	0.95
	Ferulic Acid	2.60	0.30	-0.48	0.83
	Syringic Acid	2.60	-0.50	-0.14	0.99
	Straw leachate	2.61	-1.89	0.52	0.96

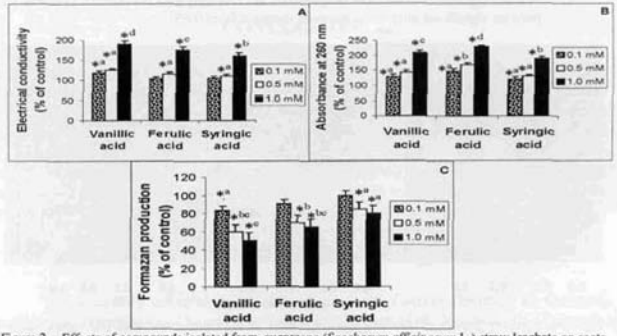

Figure 2. Effects of compounds isolated from sugarcane (*Saccharum officinarum* L.) straw leachate on roots of *Lactuca sativa* L.: leakage of (A) electrolytes and (B) non electrolytes; (C) formazan production. Bars represent the standard error of each mean and asterisks indicate significant differences at level 0.05 as compared with controls. Different letters indicate significant differences between means at level 0.05.

Phenolic acids affected the cell division in *L. sativa* root meristems (Figure 3). Vanillic acid significantly reduced the mitotic index with respect to MES buffer solution. Concentration of 1 and 0.5 mM FA reduced the cell division, while, 0.1 mM FA significantly stimulated the mitotic activity. Otherwise, 0.1 and 0.5 mM SA promoted cell division, while, 1 mM SA had no effect on mitosis. SA reduced the root elongation at concentrations > 1 mM implying that SA may inhibit root cell division at high concentrations. Blum *et al.* (5) suggested that the curvilinear decline of plant root elongation with the increase in phenolic acids concentration could be due to inhibition in root cell division and expansion. As per our results the identified phenolic acids affected the root elongation partially, through inhibition in cell division of meristematic root zone.

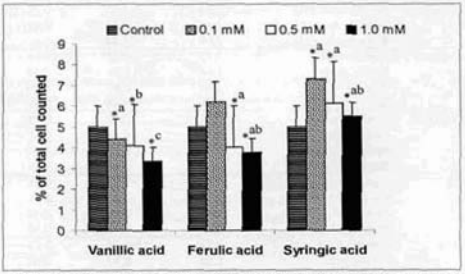

Figure 3. Mitotic Index of lettuce tips exposed to phenolic acids isolated from sugarcane (*Saccharum officinarum* L.) straw leachate. Bars represent the standard error of each mean. Asterisks indicate significant differences at level 0.05 as compared with the control. Different letters indicate significant differences between means at level 0.05.

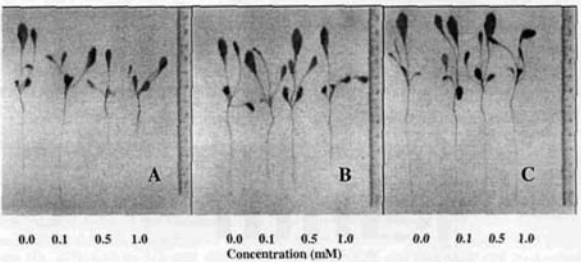

Figure 4. Seedling growth of *Lactuca sativa* L. after 30 days pretreated with (A) vanillic acid, (B) ferulic acid and (C) syringic acid of under unsterile soil.

Primary roots pretreated with VA and FA were shorter than those pretreated with MES buffer (Figure 4). Roots were less elongated and had less secondary roots as VA concentration increased. Seedlings pretreated with FA and SA increased the branching of primary roots (Figure 5A and B). Many natural monophenols can stimulate the auxin decarboxilation (6). Inhibition of auxin activity could be responsible for the observed increase in secondary roots. The observed response suggests that exposure to the isolated phenolic acids was able to modify the normal root growth pattern of *L. sativa* and that these modifications further persisted in the subsequent seedling growth.

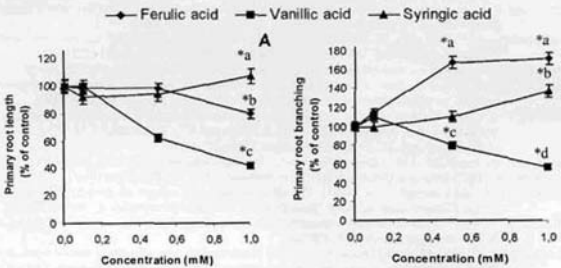

Figure 5. Elongation of primary roots (A) and number of secondary roots (B) of *Lactuca sativa* L. after 30 days pretreated with the phenolic acids isolated from sugarcane (*Saccharum officinarum L.*) straw leachate under unsterile soil. Different letters indicate significant differences between means at level 0.05.

CONCLUSIONS

The concentrations of VA, FA and SA in sugarcane straw leachates were 1.01±0.05 mmol l^{-1} (66 % *trans*; 34% *cis*), 0.82±0.02 mmol L^{-1} and 0.10±0.01 mmol L^{-1}, respectively. The root elongation of test plants and the evaluated physiological processes in *L. sativa* indicated that VA was the strongest inhibitory phytotoxin, followed by FA and SA. The relative phytotoxic potency of these compounds was different, but their effects on the physiological processes suggest that the more of action was same. As phenolic acids are often found spatially concentrated in the top soil layers under plant straw (1,4), more studies are needed to establish the participation of these compounds in field conditions.

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