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Characterization of the Allelopathic Potential of Sugarcane Leaves and Roots

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Abstract

Sugarcane cultivars that are currently planted are the result of genetic improvement focused on increased crop yield. However, this selection and genetic alteration reduced the competitive potential of sugarcane, as well as its allelopathic capabilities. Many members of the Poaceae family are highly allelopathic. Thus, the objective of this study was to characterize the allelopathic potential of two sugarcane cultivars (CTC 2 and IAC 91109) by bioassay-guided fractionation, isolation, and identification of significant phytotoxins, including those that are lipophilic. For both leaves and roots, alpha-linolenic and linoleic acid were found to be the most phytotoxic compounds found with this approach. Both compounds were phytotoxic when applied in soil and caused light-independent cellular leakage of treated cucumber cotyledon discs. We conclude that some of the phytotoxic effects of sugarcane residues in soil are due to the combined action of alpha-linolenic and linoleic acid.

Keywords

Fatty Acid, Allelochemical, Alpha-Linolenic Acid, Linoleic Acid, Bioassay, Weed

1. Introduction

Weed interference in sugarcane (*Saccharum officinarum* L.) can reduce crop yield by up to 97% [1]. However, interference is not a phenomenon caused only by weeds on crops, as the crop has the potential to limit the growth and development of weeds [2]. Allelopathy is one of the factors involved in interference

between plants.

Allelopathy is the chemical inhibition of one plant by another, due to the release into the environment of phytotoxic compounds (allelochemicals) that inhibit germination and/or growth. Plants may adversely affect growth and development of each other through the production and release of allelochemicals into the environment [3]. The Poaceae family, to which sugarcane belongs, is among the most studied plant families regarding allelopathy, producing a wide variety of allelochemicals. A necessary step in proving allelopathy is isolating and identifying the putative allelochemicals involved [4].

Some compounds produced by sugarcane can adversely affect weed communities within sugarcane fields [5]. In phytosociological surveys, Kuva *et al.* [6] [7] and Ferriera *et al.* [8] reported differences in weed infestations between areas planted with different sugarcane cultivars. This may occur due to differences in the preservation of some of the ancestors' aggressive characteristics in some cultivars, such as allelopathic potential. Viator *et al.* [9] reported postharvest sugarcane residues to be phytotoxic, and Majeed *et al.* [10] found aqueous extracts of sugarcane to be inhibitory to wheat germination and growth. Aqueous leachate of sugarcane straw can inhibit the growth of both weed and crop species [11]-[16]. The effect in these papers was largely attributed to ferulic, syringic and vanillic acids.

These studies did not look for lipophilic allelochemicals, a common oversight in many allelopathy studies [4], even though Rice [3] listed fatty acids as one class of compounds with allelopathic potential. Fatty acids of rice were shown to reduce the growth of the weeds *Heteranthera limosa* and *Echinochloa crus-galli* [17] [18]. In *Helianthus anuus* and *Helianthus tuberosus*, C10 and C18 fatty acids were associated with the allelopathic properties of these species [19]. Indeed, simple fatty acids are sufficiently phytotoxic that crude preparations of fatty acids and the nine-carbon fatty acid, pelargonic acid are sold as herbicides for organic gardening [20]. Simple fatty acids such as alpha-linolenic and linoleic acid can alter the permeability of the plant plasma membrane and disrupt chloroplast membranes [21]. Others have found alpha-linolenic and linoleic acid to be among the phytotoxic compounds produced by southern cattail (*Typha domingensis*) [22]. Recent studies have found lipophilic allelochemicals that are secondary metabolites from plants such as the very potent phytotoxin sorgoleone, which is produced in small amounts by *Sorghum* species [23] and the less potent phytotoxin aplotaxene which is produced in large amounts by *Carduus* species [24]. Thus, to properly identify all of the allelochemicals products produced by a plant species requires determination of lipophilic, as well as water-soluble phytotoxins.

The intent of this work was to partially characterize the allelopathic potential of two sugarcane cultivars (CTC 2 and IAC 911099). Yamauti [25] found aqueous extracts of CT 2, but not of IAC 911099, to inhibit *Lactuca sativa* seedling growth in a soil-free bioassay. In this study, we isolated and identified phytotoxic com-

pounds from leaves and roots of these cultivars with a bioassay-guided process that identifies the most significant phytotoxic compounds, regardless of lipophilicity.

2. Material and Methods

2.1. Plant Material and Extraction

Sugarcane mini-joints were used to produce seedlings of two cultivars (CTC 2 and IAC 911099) in sand. After sprouting and the beginning of root growth, the seedlings were transferred to soil (dystrophic Red Latosol of medium texture) in pots with a 5.0 L capacity. The plants grew at room temperature (average 21.2˚C, maximum 30.7˚C, relative humidity 60.4%) from May to September 2018 in a greenhouse in Jaboticabal, Brazil. After 150 days, fresh plant leaves and roots were collected and lyophilized, providing 55.16 and 49.2 g of leaves and roots, respectively of CTC 2 and 67.75 and 17.76 g of leaves and roots, respectively, of IAC 911099. After grinding in a Wiley mill (maximum particle size: 1.5 mm), each part of the plant was extracted at room temperature using solvents of increasing polarity: hexane (500 m), dichloromethane (DCM) (700 mL), and ethanol (EtOH) (500 mL). The extractions were performed sequentially, and the plant material remained for 24 h in each solvent, followed by filtration evaporation in a vacuum. The solid material yields of CTC 2 and ICA 911099.

2.2. Bioassay of *Lactuca sativa* and *Agrostis stolonifera* without Soil

Phytotoxicity-guided bioassays of fractions were performed with lettuce (*Lactuca sativa*) and creeping bentgrass (*Agrostis stolonifera*) in 24-well plates with hexane, DCM, and EtOH extracts from leaves and roots of the two cultivars with the method of Dayan *et al.* [26]. Phytotoxicity was assessed by qualitatively comparing seed germination and seedling growth in each well after seven days, using a rating scale of 0 to 5, where 0 indicates no effect and 5 indicates complete inhibition (no germination). The experiment was replicated.

2.3. Bioactivity-Guided Fractionation of Phytotoxicity

Initially, the materials extracted that were phytotoxic to lettuce and bentgrass (extract from CTC 2 leaves with hexane and DCM; extract from CTC 2 roots with DCM; extract from IAC 911099 leaves with DCM) were separated by normal phase chromatography. The crude extract fractionation was performed by column chromatography using an Isolera One (Biotage) system (Uppsala, Sweden) equipped with a variable wavelength UV detector and an automatic fraction collector. The column was a SNAP KP-Sil Cartridge, 37 mm × 157 mm, 50 μm irregular silica, 100 g (Biotage), and a pre-packaged SNP Sample Cartridge KP-Sil, 37 mm × 17 mm (Biotage). The separation of the extracts was performed using a gradient of hexane (solvent A) and ethyl acetate (solvent B) from 0% B

per 396 mL, 0 - 25% B per 1800 mL, 25% - 50% B per 500 mL, 50 - 100 B per 300 mL, and 100% B per 407 mL. The flow rate was 50 mL·min^{-1}. Portions of 22 mL were collected in 10 × 150-mm test tubes. The fractions were collected and recombined, based on similarities in thin layer chromatography and peak UV chromatogram profiles (254 and 280 nm). The fractionation of crude extract from CTC 2 leaves with hexane resulted in 12 fractions. Fractionation of crude extract from CTC 2 leaves with DCM resulted in 15 fractions. There were 13 fractions from the DCM crude extract of roots from CTC 2. There were 20 distinct fractions from the crude extract from IAC 911099 leaves with DCM. The fractions were bioassayed for phytotoxicity as described in Section 2.2.

The fractions were analyzed by ^{1}H NMR, ^{13}C NMR, GC/MS, and GC/FID, and fatty acids present were identified as described below.

2.4. Gas Chromatography Coupled to Mass Spectrometry (GC-MS) for Compound Identification

The GC-MS analysis was performed on an Agilent 7890AGC system equipped with an XL MSD ion detector with an Agilent 5975C detector and an Agilent 7693 autosampler. A DB-5 fused silica capillary column (30 m × 0.25 mm, with 25 μm film thickness) operated using the following conditions: injector temperature: 240˚C; column temperature: 60˚C to 250˚C at 3˚C·min^{-1} rate, then at 240˚C for 5 min; carrier gas—He; injection volume: 1 μL (25:1 split ratio); and FID temperature of 300˚C. The MS mass range was m/z 50 to 550; filament delay: 3.5 min; source temperature: 230˚C; and quad temperature: 150˚C.

2.5. Gas Chromatography Flame Ionization (GC-FID) for Fatty Acid Quantitative Analysis and Identifications

GC-FID analysis was performed on a Varian CP-3800 GC instrument. The GC was equipped with as DB-23 column (Agilent Technologies) (60 m × 25 mm capillary column, 25 μm film thickness) operated using the following conditions: injector temperature: 270˚C; column temperature: 130˚C kept for 1 min, followed by 130˚C to 170˚C at 6.5˚C min^{-1}, followed by 170˚C to 215˚C at 2.8˚C·min^{-1} and kept for 12 min followed by 215˚C to 230˚C at 40˚C min^{-1} and kept for 3 min; injection volume: 1 μL (20:1 split); 3 mL·min^{-1} constant flow; and FID temperature of 300˚C. Fatty acid methyl esters were identified by injecting commercially available standards, purchased from Sigma Aldrich, and comparing retention times with unknown times. Fatty acids were quantified by performing percentage area calculations based on the combined total area of the FID.

Free fatty acids or fractions were converted to their corresponding fatty acid methyl esters using diazomethane and direct methylation prior to GC-FID analysis. For this, 1 mg of compound/fraction in 1 mL of diethyl ether was treated at room temperature overnight with a solution of diazomethane in diethyl ether. The solvent and residual CH_2N_2 were removed using N_2, and the sample was resuspended in DCM for GC analysis.

2.6. Quantitative Bioactivity-Guided Fractionation of Phytotoxicity

The quantity of fatty acids in leaves and roots of the cultivars was determined following the method of Wang *et al.* [27] using direct methylation. An internal standard was prepared with tricosanoic acid (C23:0).

2.7. Soil Fatty Acid Bioassays

Two substrates were used in these bioassays. One substrate was s clayey, silty soil collected in a field that was never treated with herbicides at the USDA Jamie Whitten Research Center in Stoneville, Mississippi, USA (33˚26'22.68"N, 90˚53'52.9"W). The sold characteristics were: 38.5% sand, 47.75% silt 13.75% clay, 1.08% organic matter, pH 6.4, 35 $\mu g \cdot g^{-1}$ Mg, 8.2 $\mu g \cdot g^{-1}$ K, and 92.8 $\mu g \cdot g^{-1}$ Ca. The other soil used was a medium-textured dystrophic Red Latosol collected at UNESP Research Farm, Jaboticabol, SP, Brazil. The soils were air dried, sieved (0.5 mm), and stored at room temperature.

Linoleic acid (18:2n6c) and alpha-linolenic acid (18:3n3), purchased from Sigma Aldrich, were dissolved in acetone and applied to the dry soil to provide appropriate concentrations (0.33, 1, 3.33, 10, and 33.3 mM), solvent control (acetone), and positive control (0.1 and 1.0 mM atrazine). The soil was dried at room temperature to remove acetone. Soil without application was used as a control. In each well, 0.2 mL of test solution and 0.6 g of soil were added. *L. sativa* and *A. stolonifera* were sown in the clayey, silty clay soil. *L. sativa* and *Solanum lycopersicum* were sown in the dystrophic Red Latosol of medium texture. The amount of seeds used in each cell was approximately five seeds of *L. sativa*, 10 mg (approximately 115 seeds) of *A. stolonifera*, and six seeds for *S. lycopersicum*. The plates were incubated at 26˚C in a Conviron growth chamber at 173 $\mu mol \cdot s^{-1} \cdot m^{-2}$ photosynthetically active radiation. Then, 300 µL of distilled deionized water (DDI) were added on the first day and another 100 µL were added on the fourth day. To maintain humidity, the trays were covered with plastic chambers. At seven days after sowing, root length and fresh mass were determined for *L. sativa*, and the average shoot length of *A. stolonifera* was determined. The results were subjected to analysis of variance by F test, and the means were compared by Tukey test at 5% probability.

2.8. Cellular Leakage Bioassay

The effects of linoleic acid and alpha-linolenic acid on cell leakage of cucumber cotyledon discs (*Cucumis sativus* L.) were determined by the method of Dayan and Watson [28]. For linoleic acid (18:2n6c) and alpha-linolenic acid (18:3n3), purchased from Sigma Aldrich, the concentrations tested were 3.3, 10, 33, 100, 330, and 1000 µM. There was a solvent control (acetone 1%), and a positive control (50 µM acifluorfen). Measurements were made using an electrical conductivity meter (Model 1056, Amber Science, Eugene, OR 97402) equipped with an 858 Conductivity Macro Flow cell at the beginning and end of a 18-h dark incu-

bation period. Samples were measured at 1, 2, 3, 4, and 8 h after exposure to light. Each sample was replicated three times. Maximum conductivity (a positive control) was measured by boiling three samples from each treatment for 20 min. The results were subjected to analysis of variance by F test, and the means were compared by the Tukey test at 5% probability.

3. Results and Discussion

3.1. Fractionation-Guided Bioassay of Leaf and Root Extracts

The hexane and DCM crude extracts from the leaves and the DCM crude extract from the roots of CTC 2 were phytotoxic at 1 mg·mL^{-1}. All had a phytotoxicity score of 3 for *A. stolonifera* (Table 1). After fractioning these extracts with column chromatography, the fractionation of the hexane extract from leaves resulted in 12 fractions, two of which had phytotoxicity (fractions A and B). For the fractionation of dichloromethane from leaves, there were 15 fractions, only one of that was phytotoxic (fraction C). For the DCM extract of roots, there were 13 fractions. Three of them had a phytotoxic effect (fractions D, E, and F) (Table 1).

The hexane and DCM crude extracts from the leaves of IAC 911099 were phytotoxic at 1 mg·mL^{-1}. All had a phytotoxicity score of 3 for *A. stolonifera* (Table 2). The fractionation of DCM crude extracts from the leaves of IAC 911099 with column chromatography resulted in twenty distinct fractions. Six of them were phytotoxic (fractions G to L) (Table 2).

The fractions were subjected to analysis by ^{1}H NMR, ^{13}C NMR, GC/MS and GC/FID. Seven fatty acids were identified. Fatty acids were bioassayed for

Table 1. Bioassay-directed fractionation demonstrating the phytotoxicity of "CTC 2" to *Lactuca sativa* and *Agrostis stolonifera*.

Plant part (g)	Extraction Solvent/Fraction	Weight (g)	Yield (%)	Toxicity[a]	
				L. sativa	*A. stolonifera*
Leaves (55.16)	Hex	0.859	1.56	0	3
	DCM	0.678	1.23	1	3
	EtOH	1.452	2.63	0	0
Roots (49.32)	Hex	0.058	0.12	0	0
	DCM	0.190	0.39	1	3
	EtOH	1.255	2.54	0	0
Leaf fractions Hex (0.859)	Fraction A	0.0480	5.59	1	3
	Fraction B	0.0349	4.06	3	3
Leaf fractions DCM (0.678)	Fraction C	0.0200	2.95	5	0
Root fractions DCM (0.190)	Fraction D	0.0447	23.53	1	2
	Fraction E	0.0578	30.42	2	3
	Fraction F	0.0112	5.89	0	2

[a]Values denote toxicity at 1.0 mg/mL for *L. sativa* and *A. stolonifera*. 0 = no effect, 5 = maximum effect. Hex: hexane; DCM: dichloromethane; EtOH: ethanol.

Table 2. Bioassay-directed fractionation demonstrating the phytotoxicity of IAC 911099 to *Lactuca sativa* and *Agrostis stolonifera*.

Part (g)	Extraction Solvent/Fraction	Weight (g)	Yield (%)	Toxicity[a]	
				L. sativa	*A. stolonifera*
Leaves (67.45 g)	Hex	0.620	0.92	0	2
	DCM	0.795	1.18	0	3
	EtOH	2.366	3.50	0	1
Roots (17.76 g)	Hex	0.093	0.52	0	1
	DCM	0.087	0.49	0	0
	EtOH	0.846	4.76	0	0
Leaves Fractions DCM (0.795)	Fraction G	0.0341	4.29	4	4
	Fraction H	0.0093	1.17	1	3
	Fraction I	0.0274	3.45	3	2
	Fraction J	0.0295	3.71	1	3
	Fraction K	0.0225	2.83	1	2
	Fraction L	0.3448	43.37	0	3

[a]Values denote toxicity at 1.0 mg/mL for *L. sativa* and *A. stolonifera*. 0 = no effect, 5 = maximum effect. Hex: hexane; DCM: dichloromethane; EtOH: ethanol.

Table 3. Bioassay showing fatty acid toxicity to *Lactuca sativa* and *Agrostis stolonifera*.

Compound	Solvent	Toxicity[a]	
		L. sativa	*A. stolonifera*
Myristic acid	Acetone	0	0
Palmitic acid	DCM	0	0
Palmitoleic acid	Acetone	0	0
Stearic acid	DCM	0	0
Oleic acid	Acetone	0	0
Linoleic acid	Acetone	0	3
Alpha-linolenic acid	Acetone	3	3

[a]Values denote toxicity at 1.0 mg/mL for *L. sativa* and *A. stolonifera*. 0 = no effect, 5 = maximum effect. DCM: dichloromethane.

phytotoxicity (Table 3).

3.2. Identification of Compounds

^{1}H NMR and ^{13}C NMR analysis was performed on the extracts indicating a mixture of fatty acids, primarily linoleic (18:2), and alpha-linolenic (18:3). GC/MS as well as GC/FID analysis was performed for specific unequivocal identification of fatty acids. Fractions of CTC 2 and IAC 911099 leaves and CTC 2 roots contained compounds common to both cultivars. The fatty acids detected were myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linole-

ic (18:2), and alpha-linolenic (18:3). These were assessed for phytotoxicity. Linoleic acid and alpha-linolenic acid were toxic to *L. sativa* and *A. stolonifera* (Table 3). These acids are the majority in almost all fractions compared to other fatty acids (Table 4).

In the analysis of fatty acid quantification, there was no significant difference for the concentration of linoleic acid between cultivars and leaves and roots. Regarding the concentration of alpha-linolenic acid, it was higher in leaves than in roots for both cultivars. For both CTC 2 and IAC 911099, there was a higher concentration of alpha-linolenic acid in leaves and a higher concentration of linoleic acid in roots (Table 5). The concentration of 33.3 mM of alpha-linolenic acid in the soil reduced the root length of *L. sativa* seedlings in relation to the others. All treatments differed from the absolute control. The fresh mass decreased at 10 mM, similar to atrazine. Linoleic acid stimulated growth at lower concentrations and provided a fresh mass similar than that of the control at concentrations of 0.33 to 10.0 mM; only the concentration of 33.3 mM reduced fresh mass, being more effective than atrazine (Table 6).

For the bioassay of *A. stolonifera* in soil, there was a reduction in average

Table 4. Area percentage of linoleic acid and alpha-linolenic acid in each fraction.

		Linoleic acid (%)	Alpha-linolenic acid (%)
CTC 2	Fraction A	25.50	43.47
	Fraction B	10.36	55.95
	Fraction C	28.31	34.31
	Fraction D	24.28	40.43
	Fraction E	9.01	1.71
	Fraction F	16.79	19.87
IAC 911099	Fraction G	11.15	71.77
	Fraction H	8.57	85.01
	Fraction I	5.10	54.33
	Fraction J	ND*	ND
	Fraction K	ND	ND
	Fraction L	ND	ND

*ND: not detected.

Table 5. Quantitative analysis ($mg \cdot g^{-1}$ dry wt) of linoleic acid (LA) and alpha-linolenic acid (ALA) in leaves and roots of CTC 2 and IAC 911099.

	CTC 2		IAC 911099	
	Leaves	Roots	Leaves	Roots
C18:2n6c (LA)	1.06 Ba	0.90 Aa	1.16 Ba	0.99 Aa
C18:3n3 (ALA)	1.65 Aa	0.13 Bb	1.52 Aa	0.20 Bb

Means followed by the same letters, uppercase in columns and lowercase in rows, do not significantly differ by Tukey test at 5% probability.

shoot length with the increase of concentrations for both compounds. For alpha-linolenic acid, the decrease occurred at concentrations higher than 1 mM (Table 7). For linoleic acid, inhibition occurred at concentrations higher than 3.33 mM.

Table 6. Effects of alpha-linolenic and linoleic acids applied to the soil on root length (mm) and total fresh mass (mg) of seedlings of *Lactuca sativa*, at 7 days after sowing, in a clayey soil of silty clay (Mississippi, USA).

	Alpha-linolenic acid		Linoleic acid	
	Root length (mm)	Total fresh mass (mg)	Root length (mm)	Total fresh mass (mg)
Control	21.09 A	43.91 A	21.09 A	43.91 AB
Relative control	13.24 B	41.91 A	13.24 B	41.91 AB
0.1 mM atrazine	13.20 B	35.14 AB	13.20 B	35.14 B
1.00 mM atrazine	8.57 C	36.23 AB	8.57 C	36.22 B
0.33 mM	11.90 BC	40.79 A	20.93 A	47.49 A
1.00 mM	12.10 BC	42.22 A	20.03 A	50.00 A
3.33 mM	12.40 BC	42.48 A	19.10 A	40.65 AB
10.0 mM	11.70 BC	30.05 B	19.23 A	43.62 AB
33.3 mM	0.90 D	5.09 C	3.37 D	17.72 C
CV (%)	19.30	14.88	14.99	14.91
F	32.48**	32.28**	44.03**	15.50**

Means followed by the same letters, uppercase in columns, do not significantly differ by Tukey test at 5% probability. **Significant at 1% by the F test.

Table 7. Effects of alpha-linolenic acid and linoleic acid applied to the soil on average shoot length (mm) of *Agrostis stolonifera*, at seven days after sowing, in a silty clay soil (Mississippi, USA).

	Alpha-linolenic acid	Linoleic acid
Control	17.17 A	17.17 A
Relative control	17.17 A	17.17 A
0.1 mM atrazine	13.67 C	13.67 B
1.0 mM atrazine	11.83 D	11.83 C
0.33 mM	17.50 A	17.50 A
1.00 mM	17.50 A	16.83 A
3.33 mM	15.17 B	16.33A
10.0 mM	12.83 CD	13.17 B
33.3 mM	3.50 E	4.83 D
CV (%)	4.61	4.38
F	290.88**	257.77**

Means followed by the same letters, uppercase in columns, do not significantly differ by Tukey test at 5% probability. ** Significant at 1% by the F test.

In a different soil, 0.33 mM alpha-linolenic acid in the soil reduced the shoot length of seedlings of *L. sativa* dramatically (Table 8). For root length and germination, reductions occurred only at the highest concentration (33.3 mM). Linoleic acid stimulated the growth of shoots at the lowest concentrations. Allelochemicals are known to stimulate plant growth at subtoxic concentrations [29] [30]. Viator *et al.* [9] found crude sugarcane residue extract to enhance sugarcane bud germination at low doses, but to be autotoxic at higher doses, but to be autotoxic at higher doses. Linoleic acid reduced growth only at the concentration of 33.3 mM.

A concentration of 33.3 mM of alpha-linolenic acid in the soil reduced the shoot length of *S. lycopersicum* seedlings, and there was no statistical difference between it and the highest dose of atrazine (Table 9). For root length, only the highest concentration differed from that of the other treatments because, although the other concentrations differ from the control, they were equal to the relative control (acetone). The same effects also occurred for linoleic acid. However, there was a reduction in germination at the concentration of 33.3 mM. The differences in activity in the different soils, is not surprising, as Hiradate *et al.* [31] reported considerably variation in the phytotoxicity of the same allelochemical in different soils. The combined concentration of these two fatty acids in leaf material is close to 7 mM (Table 5). Our soil phytotoxicity studies (Tables 6-9) indicate that such a concentration would adversely affect the growth of some species in some soils. Such lipophilic compounds are more likely to adhere to soil particles and, thus, much less likely to leach from soil with rainfall, as would be expected with many water-soluble compounds.

Both alpha-linolenic acid and linoleic acid caused electrolyte leakage in cucumber discs at concentrations of 330 and 1000 μM after 26 h of exposure (18 h

Table 8. Effects of alpha-linolenic acid and linoleic acid applied to the soil on average shoot length and roots (mm) and germination of *Lactuca sativa* at 7 days after sowing in a dystrophic Red Latosol soil (São Paulo—Brazil).

	Alpha-linolenic acid			Linoleic acid		
	Shoot length	Root length	Germination	Shoot length	Root length	Germination
Control	21.37 a	29.70 a	4.25 a	21.37 b	29.70 a	4.25 a
Relative control	20.25 a	19.12 abc	4.50 a	20.25 b	19.12 ab	4.50 a
0.1 mM atrazine	21.47 a	25.82 ab	4.32 a	21.47 b	25.82 a	4.32 a
1.0 mM atrazine	17.22 b	16.40 bc	4.67 a	17.22 b	16.40 ab	4.67 a
0.33 mM	0.00 c	25.40 ab	4.75 a	30.77 a	25.17 a	4.00 a
1.0 mM	0.45 c	26.80 ab	5.00 a	29.77 a	23.90 a	3.75 a
3.33 mM	0.00 c	25.05 ab	4.50 a	31.72 a	24.12 a	4.00 a
10 mM	0.00 c	10.52 cd	4.50 a	15.72 b	16.80 ab	4.00 a
33.3 mM	0.00 c	0.00 d	0.00 b	0.00 c	9.37 b	1.50 b
CV (%)	10.61	24.32	16.57	13.84	26.91	19.6
F	495.77**	15.68**	20.94**	45.34**	4.81**	6.09**

Table 9. Effects of alpha-linolenic acid and linoleic acid applied to the soil on average shoot length and roots (mm) and germination of *Solanum lycopersicum* at seven days after sowing in a dystrophic Red Latosol (São Paulo—Brazil).

	Alpha-linolenic acid			Linoleic acid		
	Shoot length	Root length	Germination	Shoot length	Root length	Germination
Control	23.52 a	23.60 a	4.75 a	23.52 a	23.60 a	4.75 a
Relative control	21.00 a	13.47 c	4.75 a	21.00 ab	13.47 b	4.75 a
0.1 mM atrazine	24.92 a	24.05 a	4.75 a	24.92 a	24.05 a	4.75 a
1.0 mM atrazine	10.22 b	19.22 b	2.75 b	10.22 bc	19.22 ab	2.75 b
0.33 mM	24.85 a	14.27 c	4.25 a	25.30 a	12.72 b	4.75 a
1.0 mM	26.35 a	13.27 c	4.75 a	23.57 a	14.70 b	4.50 a
3.33 mM	27.92 a	13.00 c	4.25 a	20.45 ab	14.97 b	4.00 ab
10 mM	23.80 a	14.07 c	4.75 a	17.45 ab	14.42 b	4.50 a
33.3 mM	9.60 b	8.15 d	4.50 a	0.00 c	13.30 b	2.75 b
CV (%)	20.69	7.9	11.81	24.6	16.22	17.4
F	9.38**	70.89**	6.31**	13.54**	10.74**	5.35**

of darkness, followed by 8 h of light) (Figure 1 and Figure 2). The effects were not light dependent, as is the case for acifluorfen, a herbicide that causes phytotoxicity by causing the accumulation of the photodynamic compound protoporphyin IX [32]. Unlike acifluorfen, the two compounds caused leakage in darkness, and the effect seemed to be reduced by exposure to light. Thus, these compounds might cause phytotoxicity in soil, where there is little or no light.

Fatty acids are ubiquitous in plants. In sugarcane, palmitic and linoleic acids occur in culms and leaves and stearic and oleic acids occur in culm wax [33]. A DCM extract from sugarcane leaves containing fatty and phenolic acids showed a deleterious effect on the weed *Calopogonium mucunoides* [34]. It inhibited germination (35%), root growth (52.8%), and hypocotyls (47.1%). These previous data support those found for the sugarcane cultivars CTC 2 and IAC 911099. Fatty acids occurred in the fractions of leaves extracted using dichloromethane and in the fraction with hexane for CTC 2. Fatty acids also occurred in the DCM fraction of the roots of CTC 2. In wheat, there are three main phytochemical categories that can cause allelopathic effects, namely phenolic, hydroxamic, and short-chain fatty acids [35]. Fatty acids inhibit the growth of wheat seedlings [36] [37].

In *Typha latifolia*, a perennial monocot, there were unsaturated fatty acids, alpha-linolenic acid, and linoleic acid [22] [38]. Despite being common compounds in plants, they caused inhibition in algae similar as that of $CuSO_4$ (0.5 μmol). Alpha-linolenic acid caused the greatest algae inhibition.

There was an inhibitory effect on the growth of the weed *Echinochloa crus-galli*, due to 50 ppm fatty acids in rice husk extracts (*Oryza sativa*) from [39]. Fatty

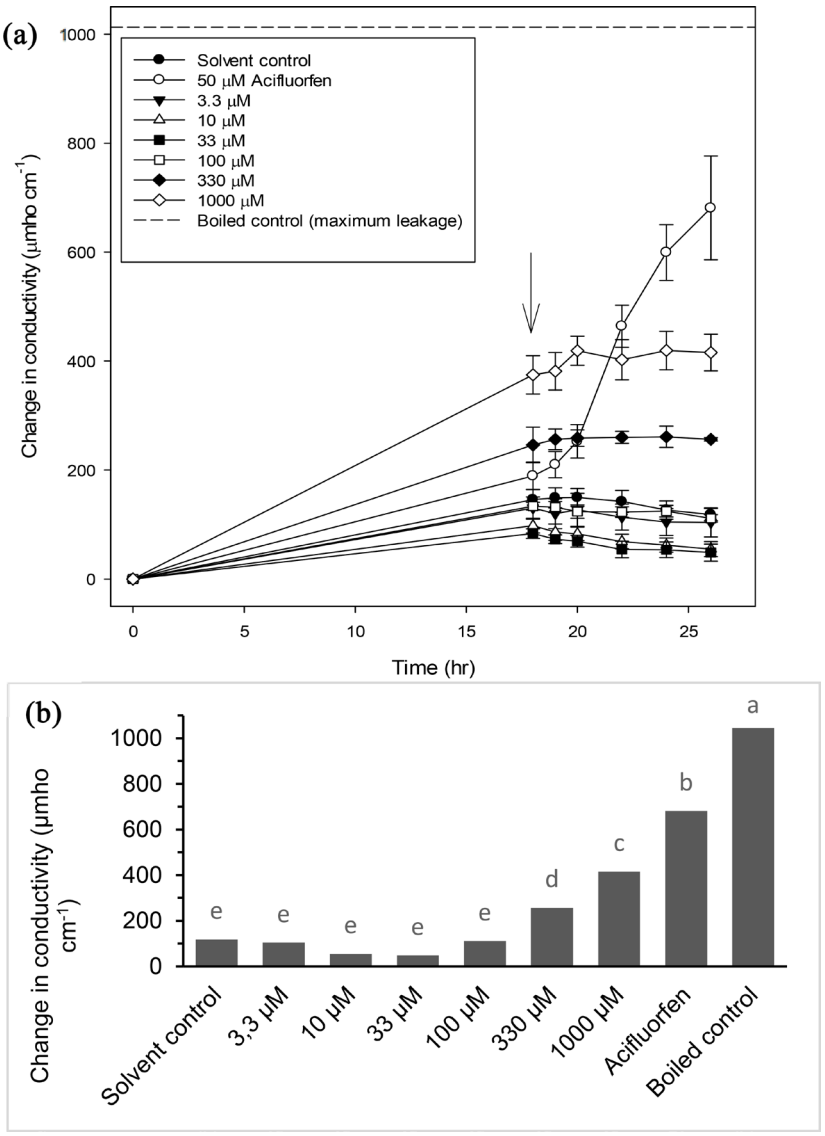

Figure 1. Effects of alpha-linolenic acid concentrations on the leakage of electrolytes from cucumber cotyledon discs during the dark 18 h. (a) Effects at 0, 18, 19, 20, 22, 24, and 26 h. The dotted line is the maximum level of leakage possible. (b) Effects at 26 h. Means followed by the same letter do not differ significantly by Tukey test at 5% probability.

acids are among the compounds with the greatest herbicidal potential according to Macías [40]. In the present study, by observing the effects of alpha-linolenic and linoleic acids on both *L. sativa* and *A. stolonifera*, there was a reduction in the characteristics analyzed in relation to the control at concentrations near 10^{-3} M. This means that, for inhibition to occur, the concentration should be higher than that indicated by Macías [40].

For *L. sativa* germinated in soil, there was a greater reduction in fresh mass compared to the control for the treatment with alpha-linolenic acid (88.4% at the highest concentration) than for the linoleic acid (59.6%) (**Table 6**). This may have occurred due to the greater amount of double bonds in alpha-linolenic acid.

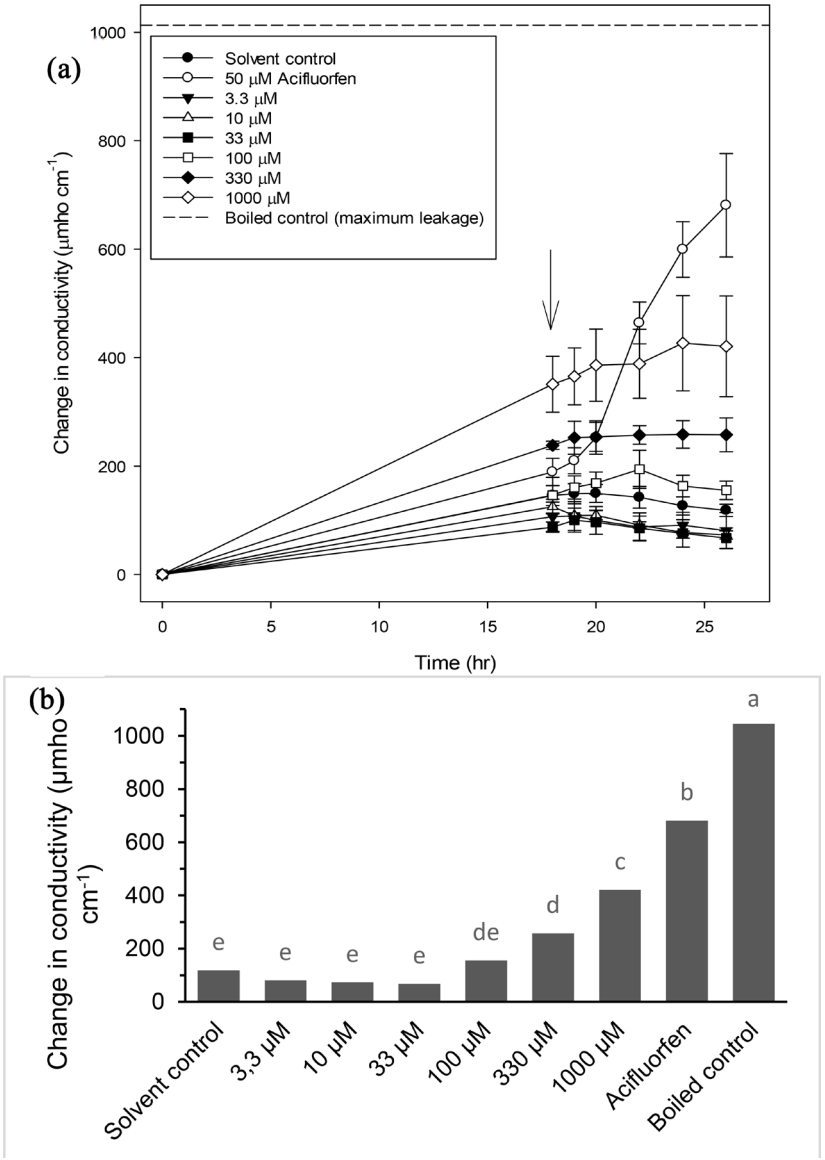

Figure 2. Effects of linolenic acid concentrations on the leakage of electrolytes from cucumber cotyledon discs during the dark 18 hours. (a) Effects at 0, 18, 19, 20, 22, 24, and 26 h. The dotted line is the maximum level of leakage possible. (b) Effects at 26 h. Means followed by the same letter do not differ significantly by Tukey test at 5% probability.

Unsaturated fatty acids have a greater phytotoxic activity [41] [42].

Medium chain fatty acids (those between nine and 11 carbons) cause damage to bimolecular lipid membranes. It makes the membrane structure unstable and allows leakage of electrolytes from the cell, culminating in plant death. This damage caused to the plant membrane can occur due to hydrophobicity of fatty acids. Hydrophobicity increases as the carbon chain of fatty acids increases [43]. In the present study, the leakage of electrolytes from cucumber cells occurred with exposure to alpha-linolenic acid and linoleic acid at 330 and 1000 μM. Concentrations below these did not differ statistically from solvent control. However, even the highest concentrations of these fatty acids did not cause the level of

cellular leakage that acifluorfen caused, which promotes loss of plasma membrane integrity after exposure of samples to light, due to production of high levels of reactive oxygen species generated by accumulation of the photodynamic compound protoporphyrin IX [28]. The importance of monitoring the plasma membrane stems from its role in the interface between the cell and the environment. If there is sufficient loss of the integrity of the lipid bilayer, the leakage of electrolytes will result cell death [28].

In a search for bioactive compounds from the plant *Ligularia macrophylla*, both linoleic and alpha-linolenic acids were isolated [44], but they had little or no phytotoxicity in a bioassay for which the highest concentration was 3.4-fold lower than the initial bioassay used in this paper. However, both compounds were antifungal.

Both linoleic and alpha-linolenic acids are ubiquitous compounds in plants, so one could argue that they are unlikely to influence other plant species as allelochemicals. However, as the father of toxicology, Paracelsus, deduced almost five centuries ago, "the poison is in the dose". We have found these compounds are phytotoxic at relatively high doses and that they retain their phytotoxicity in soil, something that is critical for an allelochemical. The simple phenolic acids previously reported as allelochemicals from sugarcane [11]-[16] are virtually inactive in most soils [45]. Our results are consistent with those of Luz *et al.* [46], who found the DCM and ethyl acetate fractions of sugarcane vinasse to be the most phytotoxic to lettuce seedling root growth. Many compounds touted as allelochemicals because of activity in soil-free bioassay have little or no activity in soil [4] [31] [45]. In some soils of sugarcane, there could be high enough combined concentrations of these compounds in soil to inhibit the growth of some weed species.

4. Conclusion

Alpha-linolenic acid and linoleic acid were found and identified as potential allelochemicals from leaves and roots of the sugarcane cultivars CTC 2 and IAC 911099. These compounds caused growth inhibition of *Lactuca sativa*, *Agrostis stolonifera*, and *Solanum lycopersicum* seedlings and cellular leakage of *Cucumis sativus* cotyledon discs in darkness. Furthermore, both compounds were active in inhibiting seedling growth in soil, a prerequisite for allelochemical activity.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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