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Allelopathic Potential of Plant Aqueous Mixtures on *Euphorbia heterophylla*

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Abstract: *Euphorbia heterophylla* is a widely distributed weed whose seeds can remain viable in the soil for years, competing with crops. Therefore, natural herbal preparations could be a solution for its more diversified management. This study investigates the efficacy and mode of action of aqueous mixtures of *Urochloa ruziziensis* stems and *Sorghum bicolor* roots and stems on *E. heterophylla* seed germination, seedling development, antioxidant enzyme activity, and respiration. Aqueous mixtures with concentrations of 0 (control), 250, 500, 750 and 1000 ppm were prepared. *E. heterophylla* seeds were treated with the mixtures and incubated under controlled conditions. Seedling development, respiration, and enzyme activity were assessed after 4 days of incubation and germination was analyzed after 16 days. *Urochloa ruziziensis* and *S. bicolor* mixtures presented allelopathic effects on *E. heterophylla* inhibiting root growth, root fresh and dry weights and induced mitochondrial alterations resulting in oxidative stress, increasing the antioxidant enzymes catalase and peroxidase. *U. ruziziensis* and *S. bicolor* aqueous mixtures were found to have potential in controlling the weed *E. heterophylla* in laboratory tests.

Keywords: allelopathy; *Brachiaria*; *Sorghum*; mitochondria; oxidative stress

1. Introduction

Euphorbia heterophylla, commonly known as wild poinsettia, milkweed, and painted euphorbia, is a weed of difficult management affecting agricultural crops throughout South America, Africa, Asia, and Australia [1]. The plant infests annual as well as perennial crops, and herbicide resistant populations are common [1–3]. Therefore, novel and effective methods of control are required. In northwestern Paraná State, Brazil, *E. heterophylla* directly affects corn and soybean, increasing management costs and yield losses [4–6]. Thus, natural herbal preparations could be a solution for the more diversified management of *Euphorbia heterophylla*.

Allelopathy, a phenomenon by which organisms interfere in the development of other living beings, can be used as a tool to control weeds [7]. Research in allelopathy show that it can be used to control weeds and to reduce synthetic chemical input in agriculture. A large number of plant and weed species produce secondary metabolites known as allelochemicals. They can be used to control weeds in agricultural systems by using allelopathic crops for intercropping, crop rotation, or mulching. A few important examples of crop species with high allelopathic potential are wheat, rice, sorghum, rye, barley, and sunflower [8].

Among the tested species and varieties of rice, several have been found to have allelopathic effects on other plants and, therefore, the potential to be used for weed control in agriculture [9]. According to Dhungana [10], weed suppression by intercropping is basically attributed to increasing competition between the crop plants and the weeds and/or the allelopathy effect of some crop plants. Therefore, the effect of root extracts of maize or soybean on *Bidens* sp. and *Eleusine* sp. weeds, as well as the effect of sole cropping of corn or soybean on weed occurrence and growth were efficient in controlling them.

Allelopathic chemicals have been found in root exudates, leachates, leaf volatiles and decomposing plant material. They can belong to several chemical classes, such as phenolic acids, flavonoids, lactones, ketones, coumarins, alcohols, polyphenols, glycosides, alkaloids, aldehydes, and terpenes. Understanding the importance of allelochemicals for interactions between weeds and crops, weeds and weeds, weeds and plant pathogens and weed autotoxicity are keys for successful weed management without herbicides. In addition, weed allelochemicals and the syntheses of their derivatives may have potential in the development of bioactive pesticides [11].

An example of allelopathic interactions between crops and weeds can be seen in no-till systems—a crop management strategy widely used in Brazil because of its long-term benefits to soil quality and agricultural production. Allelopathic chemicals are released during the decomposition of green manure, affecting weed growth and germination [12]. Allelopathy shows potential as an innovative control strategy capable of reducing the environmental impacts caused by excessive herbicide use and increasing agricultural sustainability. The efficacy of this strategy depends on the capacity of beneficial plant species to produce allelopathic chemicals in sufficient concentrations to inhibit the development of harmful species [13].

Previous studies have shown that *Urochloa ruziziensis* (R. Germ. and CM Evrard) Crins (basionym *Brachiaria ruziziensis*), when used as cover crop, reduces the emergence of certain plants and weeds in the field [14–18]. According to Moreno et al. [18], eight compounds were isolated from *U. ruziziensis*: friedelin, oleanolic acid, α -amyrin, 1-dehydrodiosgenone, sitosterol and stigmasterol glycosides, triclin and *p*-coumaric acid. The phytotoxic effects of crude methanolic extract and fractions of ruzigrass were assessed using germination rate, initial seedling growth, and biomass of *Bidens pilosa*, *Euphorbia heterophylla* and *Ipomoea grandifolia*, as *B. pilosa* was the most affected by fractions of ruzigrass.

Urochloa spp. occupy about 100 million hectares of pasture area in Brazil [19]. Their suppressive effects are likely related to the release of water-soluble allelochemicals in the soil [20]. Another cover crop species, *Brachiaria plantaginea*, was found to inhibit the germination of tropical spiderwort (*Commelina benghalensis*), *E. heterophylla*, and *Ipomoea grandifolia* in soybean fields [12,21]. When planted as cover crops, millet and *U. ruziziensis* show potential to suppress the emergence and initial growth of weeds [22].

Sorghum has one of the greatest allelopathic activities against weeds [23,24]. Phenolic acids are released during sorghum decomposition promoting short-term suppression of weeds [25,26]. According to Cheema and Khaliq [27], Sorghum is well recognized for its allelopathic effects on other crops. Mature sorghum plants possess a number of water soluble allelochemicals (nine) (for example: sorgoleone, cyanogenic glycosides-dhurrin, and a number of breakdown products of phenolics) which are phytotoxic to the growth of certain weeds such as *Phalaris minor* Retz., *Chenopodium album* L., *Rumex dentatus* L. and *Convolvulus arvensis* L. Furthermore, *Sorghum allelopathy* can be used as sorgaab (water extract of mature *Sorghum bicolor* L. Moench plants obtained after soaking in water for 24 h and sprayed as a natural herbicide), sorghum mulch, sorghum soil incorporation and in crop rotation.

Weston et al. [28], observed the allelopathic effects of sorghum residues on various weeds in monocrop and intercrop systems. Another report found that the use of both *U. ruziziensis* and sorghum as cover crops in soybean fields enhanced the allelopathic effects against weeds because of their increased plant cover and biomass generation [29].

Although *U. ruziziensis* and *Sorghum bicolor* L. Moench have been shown to exert inhibitory effects on the germination and growth of various weeds, including *E. heterophylla* [6,30,31], little is known about the biochemical effects of their allelochemicals on target weeds, particularly on mitochondrial

metabolism. Given the herbicidal and growth regulatory effects of allelochemicals, added to the competition generated by allelopathic plants against weeds, it is crucial to identify which molecules act as allelochemicals and understand their action on plant cells. This study aimed to investigate the effects and mode of action of aqueous mixtures of *U. ruziziensis* and *S. bicolor* on *E. heterophylla* seed germination, seedling growth, antioxidant enzyme activity, and respiration.

2. Materials and Methods

2.1. Location

The research was carried out at the Laboratory of Biochemistry and the experimental farm (CAU-Umuarama campus, Umuarama, Brazil) and the Laboratory of Biological Oxidation (Maringá, Brazil) of the State University of Maringá, Paraná, Brazil.

2.2. Weed Seeds

Seeds of *E. heterophylla* were collected from the Caiuá sandstone region of Paraná State in 2018. The seeds were stored at a temperature of 10 °C/dark for 1 month.

2.3. Plant Material and Extract Preparation

Seeds of *S. bicolor* cv. BRS 506 and *U. ruziziensis* were collected from the Caiuá sandstone region of Paraná State in 2018. They were planted on the experimental farm (Umuarama campus), (23°47'28" S, 53°15'22" W and elevation: 340 m). After they were grown from seed, they were harvested, dried, and extracted in January 2018.

S. bicolor plants were harvested at 60 days after emergency, that is, in the pre-flowering stage [29]. After collection, the plant material was separated into roots and stems. Both fractions were oven-dried to constant weight for 5 days at 65 °C and ground. The resulting powder was mixed with distilled water to obtain mixtures concentrations of 250, 500, 750, and 1000 ppm (parts per million). Then, the mixtures were left to stand for 24 h/dark at room temperature (25 °C), filtered through filter paper, and used as aqueous mixtures [32]. Distilled water was used as control.

Urochloa ruziziensis plants were harvested at 60 days after emergence, and separated into aerial parts. The stems were oven-dried to constant weight for 3 days at 65 °C and ground. Then, the resulting powders were mixed with distilled water, left to stand for 24 h/dark at room temperature (25 °C), and filtered to obtain mixtures at 250, 500, 750, and 1000 ppm (parts per million) Distilled water was used as control.

2.4. Seed Germination Studies

In the laboratory, *E. heterophylla* seeds were sterilized with 1% sodium hypochlorite solution, washed with distilled water, and placed, in groups of 50, in a transparent plastic box (11 × 11 × 3 cm) containing 2 sheets of germination paper, which was moistened with 10 mL of distilled water (control) or aqueous mixtures. Samples were incubated in a biochemical oxygen demand incubator at 28 °C under a 12 h/12h light/dark photoperiod for 16 days [33]. The seeds were evaluated daily for germination percentage and speed. Germination speed (GS) was calculated by the following formula [34]: $GS = (G_1/N_1) + (G_2/N_2) + \dots + (G_f/N_f)$, where G_1 , G_2 , and G_f are the number of germinated seeds in the first, second, and final count, respectively, and N_1 , N_2 , and N_n are the first, second, and final evaluation days, respectively.

During the experiment, it was observed that the first leaves appeared at 4 days after incubation, indicating that photosynthesis had begun to contribute to the energy metabolism of seedlings. Thus, subsequent experiments were carried out using 4-day-old seedlings.

2.5. Initial Seedling Growth

The development of control and mixture-treated seedlings was assessed by determining the length of the primary root and hypocotyl (primary stem). After 4 days of incubation, the primary roots and hypocotyl were removed, measured (cm), and weighed (mg) on an analytical balance to obtain the root and hypocotyl fresh weights. Then, samples were oven-dried to constant weight at 65 °C and weighed to obtain the root and hypocotyl dry weights.

2.6. Seedling Respiration

At 4 days after germination, control and mixture-treated seedlings were evaluated for respiratory activity. Oxygen consumption was measured at 25 °C using a Clark-type oxygen electrode in an acrylic chamber connected to a polarograph [35]. Root samples ($n = 6$ replicates) were cut into 5 cm segments, weighed, and immediately placed in an oxygen electrode vessel containing 2 mL of a solution (pH 5.8) of 2 mM $\text{Ca}(\text{NO}_3)_2$, 2 mM KNO_3 , 0.43 mM NH_4Cl , 0.75 mM MgSO_4 , and 20 mM NaH_2PO_4 [36], (medium free of FeCl_3 , pH adjusted to 6.5). Each measurement was performed in 6 replicate samples. The contribution of mitochondrial cytochrome oxidase (COX), alternative mitochondrial oxidase (AOX), and extramitochondrial oxidases to total respiration was estimated by adding 200 μM potassium cyanide (KCN) to the reaction medium. Oxygen uptake was monitored for 15 min. Absorption rates were calculated from polarographic records assuming an initial dissolved oxygen concentration of 240 μM at 25 °C [37]. Results are presented in relation to the root fresh weight.

2.7. Antioxidant Enzyme Activity

At 4 days after germination, root fragments (0.2 g) from control and mixture-treated seedlings were collected and homogenized in a mortar (4 °C) with 2.0 mL of extraction medium (phosphate buffer, 67 mM potassium, pH = 7.0; 2% polyvinylpyrrolidone). The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatant was used as enzyme extract for evaluation of peroxidase (POD) and catalase (CAT) activities.

For determination of POD activity, a 200 μL aliquot of enzyme extract was added to 3 mL of reaction medium (25 mM potassium phosphate buffer, pH 6.8; 10 mM H_2O_2 ; and 2.58 mM guaiacol). POD activity was measured spectrophotometrically at 470 nm using an extinction coefficient of $25.5 \text{ mM}^{-1} \text{ cm}^{-1}$ [38]. Results are expressed in millimoles of tetraguaiacol produced per minute per gram of root.

For determination of CAT activity, enzyme extract (100 μL) was added to 3 mL of reaction medium (67 mM potassium phosphate buffer, pH 7.0; 10 mM H_2O_2). CAT activity was measured spectrophotometrically at 240 nm using an extinction coefficient of $0.0394 \text{ mM}^{-1} \text{ cm}^{-1}$ [39]. Results are expressed in millimoles of peroxide consumed per minute of reaction per gram of root.

2.8. Statistical Analysis

Four replicate experiments were conducted using a completely randomized design with five mixtures concentrations and four replications. Data were subjected to regression analysis using SigmaPlot version 12.0 (Systat Software, Inc., San Jose, CA, USA), and biologically sound, significant, good-fitting (R^2) models ($p \leq 0.05$) were selected. Because not all data were satisfactorily described by the tested models, we also compared differences between means using analysis of variance followed by Tukey's or Duncan's multiple range test ($p \leq 0.05$).

3. Results

Aqueous mixtures of *U. ruziziensis* and *S. bicolor* roots and stems significantly influenced the growth parameters of *E. heterophylla* (Table 1), except for germination percentage and speed. CAT and POD activities and total respiration were influenced by extract $\times$ concentration interaction effects.

Table 1. Analysis of variance for germination percentage (GP), germination speed (GS), root length (RL), root fresh weight (RFW), root dry weight (RDW), hypocotyl length (HL), hypocotyl fresh weight (HFW), hypocotyl dry weight (HDW), catalase activity (CAT), peroxidase activity (POD), total respiration (TR), cyanide-sensitive respiration (CSR), and cyanide-insensitive respiration (CIR) of *Euphorbia heterophylla* treated with 0 (control), 250, 500, 750, and 1000 ppm aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems.

Source	df	GP	GS	RL	RFW	RDW	HL	HFW	HDW	CAT	POD	TR	CSR	CIR
A	2	0.000 **	0.004 **	0.01 **	0.043 ns	0.002 **	0.000 **	0.003 **	0.151 ns	0.000 **	0.000 **	0.000 **	0.000 **	0.001 **
B	4	0.151 ns	0.571 ns	0.000 **	0.000 **	0.000 **	0.000 **	0.016 **	0.000 **	0.000 **	0.000 **	0.000 **	0.000 **	0.002 **
A × B	8	0.259 ns	0.716 ns	0.380 ns	0.783 ns	0.883 ns	0.115 ns	0.123 ns	0.222 ns	0.000 **	0.000 **	0.020 *	0.115 ns	0.202 ns
Error	45													
Total	59													
CV (%)		9.76	14.97	14.40	16.25	18.93	15.01	18.93	15.07	21.44	21.12	10.85	16.45	14.10

A, mixture type; B, mixture concentration; CV, coefficient of variation; df, degrees of freedom; ns, not significant; * significant at $p < 0.05$; ** significant at $p < 0.01$.

3.1. Germination

Euphorbia heterophylla germination was not influenced by treatment with aqueous mixture (Table 2). The highest germination percentage (62%) was observed in the control; and the lowest (51.5%), in seeds treated with *U. ruziziensis* mixture. Control seeds showed the lowest germination speed (6.63), and seeds treated with *S. bicolor* stems mixture showed the highest germination speed (7.96), differing from other treatments.

Table 2. Germination percentage and germination speed of *Euphorbia heterophylla* treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations. Evaluations were performed at 16 days after incubation.

Treatment	Germination (%)			Germination Speed (Seeds Day ⁻¹)		
<i>U. ruziziensis</i> shoots	54.85 ^b			6.34 ^b		
<i>S. bicolor</i> roots	56.85 ^b			6.59 ^b		
<i>S. bicolor</i> stems	64.05 ^a			7.44 ^a		
<i>p</i> -value	**			**		
Concentration (ppm)	<i>U. ruziziensis</i> stems	<i>S. bicolor</i> roots	<i>S. bicolor</i> stems	<i>U. ruziziensis</i> stems	<i>S. bicolor</i> roots	<i>S. bicolor</i> stems
0	62.00	62.00	62.00	6.63	6.63	6.63
250	57.50	58.00	63.25	6.63	6.15	7.39
500	51.75	55.50	64.50	6.00	6.85	7.88
750	51.50	55.00	64.00	5.95	6.30	7.33
1000	51.50	53.75	66.60	6.49	7.04	7.96
<i>p</i> -value	ns			ns		

** Significant at $p \leq 0.01$ by ANOVA followed by Tukey's or Duncan's multiple range test ($n = 4$); ns, not significant.

^{a,b} Means within columns followed by the same lowercase letter do not differ at $p < 0.01$ by Tukey's test.

3.2. Initial Development

The three aqueous mixtures exerted a greater effect on *E. heterophylla* root length than on hypocotyl length (Table 3). Root growth was inhibited by all treatments.

Table 3. Root and hypocotyl lengths of 4-day-old *Euphorbia heterophylla* seedlings treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations.

Mixtures Concentration (ppm)	Root Length (cm)			Hypocotyl Length (cm)		
	<i>U. ruziziensis</i> Stems	<i>S. bicolor</i> Roots	<i>S. bicolor</i> Stems	<i>U. ruziziensis</i> Stems	<i>S. bicolor</i> Roots	<i>S. bicolor</i> Stems
0	4.38	4.38	4.38	2.51	2.51	2.51
250	2.99 *	3.87 *	3.11 *	1.52 *	2.43	1.99
500	2.71 *	3.19 *	3.15 *	1.54 *	2.44	2.19
750	2.50 *	2.90 *	2.98 *	1.52 *	2.10	2.31
1000	2.27 *	2.94 *	2.95 *	1.44 *	1.86	2.12

An asterisk (*) denotes a significant difference from the control (0 ppm) by analysis of variance (ANOVA) followed by Duncan's multiple range test ($p \leq 0.05$).

Urochloa ruziziensis mixture inhibited root growth at all concentrations. At 1000 ppm, the aqueous mixture inhibited root growth by 48% compared with the control. *S. bicolor* root mixture at concentrations of 500 ppm and higher inhibited root growth by about 32%. *S. bicolor* stems mixture inhibited root growth by 32%, regardless of concentration. Only *U. ruziziensis* mixture inhibited hypocotyl growth. The effects were observed at all concentrations, with the highest concentration (1000 ppm) affording the highest inhibition (43%) (Table 3).

Aqueous mixtures reduced *E. heterophylla* root fresh and dry weights (Table 4) but had little effect on hypocotyl fresh and dry weights (data not shown).

Table 4. Root fresh and dry weights of 4-day-old *Euphorbia heterophylla* seedlings treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations.

Mixtures Concentration (ppm)	Root Fresh Weight (mg)			Root Dry Weight (mg)		
	<i>U. ruziziensis</i> Stems	<i>S. bicolor</i> Roots	<i>S. bicolor</i> Stems	<i>U. ruziziensis</i> Stems	<i>S. bicolor</i> Roots	<i>S. bicolor</i> Stems
0	14.56	14.56	14.56	0.79	0.79	0.79
250	9.92 *	10.11 *	8.53 *	0.58 *	0.50 *	0.42 *
500	8.81 *	8.58 *	8.19 *	0.56 *	0.50 *	0.45 *
750	8.00 *	7.34 *	8.57 *	0.53 *	0.37 *	0.43 *
1000	8.45 *	7.77 *	6.71 *	0.48 *	0.36 *	0.34 *

An asterisk (*) denotes a significant difference from the control (0 ppm) by ANOVA followed by Duncan's multiple range test ($p \leq 0.05$).

All tested concentrations of *U. ruziziensis* stems and *S. bicolor* root mixtures produced similar effects on root weight. The *U. ruziziensis* mixture reduced root fresh and dry weights by about 38 and 29%, respectively. The *S. bicolor* root mixture reduced root fresh and dry weights by 43% compared with the control. The aqueous mixture of *S. bicolor* stems also reduced root weights. The greatest reduction was obtained using 1000 ppm: root fresh weight decreased by 43% and root dry weight by 46%.

Overall, all mixtures inhibited the initial development of *E. heterophylla*, but the greatest effects were obtained using the mixture at the highest concentration, 1000 ppm.

3.3. Root Respiration

Figure 1A–C shows the oxygen consumption rate of *E. heterophylla* seedling roots in the presence of aqueous mixtures. Mitochondrial respiration (KCN-sensitive respiration) in the control corresponded to 63% of the total respiration rate, whereas non-mitochondrial respiration (KCN-insensitive respiration) corresponded to only 37% (Figure 1). Thus, during the 4 days of incubation, the predominant type of respiration of *E. heterophylla* seedlings was mitochondrial.

The *Urochloa ruziziensis* mixture (Figure 1A and Table 5) reduced total respiration rate by 36% at all concentrations tested. KCN-sensitive respiration decreased by up to 68% at 1000 ppm. These results are in line with the reduction in root length and weights (Tables 3 and 4, respectively). KCN-insensitive respiration, in contrast, was not significantly influenced by treatment with the aqueous mixture.

Table 5. Comparison of total respiration rate ($\text{nmol min}^{-1} \text{g}^{-1}$) in *Euphorbia heterophylla* roots treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations.

Mixtures	Concentration (ppm)				
	0	250	500	750	1000
U. ruziziensis stems	309.6150 a,A	227.6025 a,B	221.1375 b,B	204.1325 b,B	198.1650 b,B
S. bicolor roots	309.6150 a,A	239.7475 a,B	265.4925 a,b,A,B	243.5975 b,B	251.3925 a,B
S. bicolor stems	309.6150 a,A	259.6575 a,A	302.0950 a,A	314.0550 a,A	284.0675 a,A

a,b,A,B Means followed by the same lowercase letter within a column or uppercase letter within a row do not differ at $p > 0.05$ by Tukey's test.

Figure 1. Respiratory activity in roots of 4-day-old *Euphorbia heterophylla* seedlings treated with aqueous mixtures of (A) *Urochloa ruziziensis* stems, (B) *Sorghum bicolor* roots, and (C) *S. bicolor* stems at different concentrations. Respiratory activity was measured in the absence (total respiration) and presence of potassium cyanide (KCN-insensitive respiration). KCN-sensitive respiration was calculated as the difference between total respiration and KCN-insensitive respiration. Each data point is the mean $\pm$ standard error. An asterisk (*) denotes a significant difference from the control (0 ppm) by ANOVA followed by Duncan's multiple range test ($p \leq 0.05$).

The *Sorghum bicolor* roots mixture (Figure 1B and Table 5) had a lower effect than *U. ruziziensis* mixture. Total and KCN-sensitive respiration were reduced by about 22% and 43%, respectively, at all mixture concentrations. KCN-insensitive respiration was not affected.

Total respiration was not influenced by treatment with *S. bicolor* stems mixture (Figure 1C and Table 5). KCN-sensitive respiration was reduced by about 39% at all concentrations. KCN-insensitive respiration, which is associated with the action of antioxidant enzymes, such as lipoxygenase and alternative mitochondrial oxidase, was stimulated in the presence of mixture. *S. bicolor* stems mixture (1000 ppm) increased non-mitochondrial respiration by 45% compared with the control. The mixture reduced root length and weight, probably by promoting oxidative stress, as evidenced by the increase in KCN-insensitive respiration.

3.4. Antioxidant Enzymes

The activity of antioxidant enzymes CAT and POD in the root of *E. heterophylla* was, in the most part, affected by aqueous mixtures (Figure 2). *U. ruziziensis* and *S. bicolor* stem mixtures increased CAT activity; the former by 2.4 times at 250 and 1000 ppm and the latter by 1.8 and 2.1 times at 250 and 1000 ppm, respectively (Figure 2A,B and Table 6). CAT activity was not affected by *S. bicolor* root mixture.

Figure 2. (A) Catalase and (B) peroxidase activities in roots of 4-day-old *Euphorbia heterophylla* seedlings treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations. Each data point is the mean $\pm$ standard error. An asterisk (*) denotes a significant difference from the control (0 ppm) by ANOVA followed by Duncan's multiple range test ($p \leq 0.05$).

Figure 2B and Table 7 show that POD activity increased by 3.7-fold in the presence of 250 and 750 ppm *U. ruziziensis* mixture, by 5.4-fold in the presence of low concentrations of *S. bicolor* root mixture, and by 9-fold in the presence of 1000 ppm *S. bicolor* root mixture. *S. bicolor* stems mixture increased POD activity by 5.6 times at all concentrations.

Table 6. Comparison of catalase activity ($\text{mM H}_2\text{O}_2$ consumed $\text{min}^{-1} \text{g}^{-1}$) in *Euphorbia heterophylla* roots treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations.

Mixtures	Concentration (ppm)				
	0	250	500	750	1000
U. ruziziensis stems	0.05402 ^{a,C}	0.1305 ^{a,A}	0.0982 ^{a,A,B}	0.0816 ^{a,B,C}	0.1295 ^{a,A}
S. bicolor roots	0.05402 ^{a,A}	0.0376 ^{c,A}	0.0599 ^{b,A}	0.0570 ^{a,A}	0.0542 ^{b,A}
S. bicolor stems	0.05402 ^{a,C}	0.0971 ^{b,A,B}	0.0654 ^{b,B,C}	0.0724 ^{a,B,C}	0.1147 ^{a,A}

^{a,b,c,A,B,C} Means followed by the same lowercase letter within a column or uppercase letter within a row do not differ at $p > 0.05$ by Tukey's test.

Table 7. Comparison of peroxidase activity (μM tetraguaiacol produced $\text{min}^{-1} \text{g}^{-1}$) in *Euphorbia heterophylla* roots treated with aqueous mixtures of *Urochloa ruziziensis* stems, *Sorghum bicolor* roots, or *S. bicolor* stems at different concentrations.

Mixtures	Concentration (ppm)				
	0	250	500	750	1000
U. ruziziensis stems	0.0039 ^{a,C}	0.0140 ^{b,A}	0.0057 ^{b,C}	0.0131 ^{b,A,B}	0.0071 ^{c,B,C}
S. bicolor roots	0.0039 ^{a,C}	0.0197 ^{a,B}	0.0205 ^{a,B}	0.0200 ^{a,B}	0.0330 ^{a,A}
S. bicolor stems	0.0039 ^{a,B}	0.0222 ^{a,A}	0.0203 ^{a,A}	0.0196 ^{a,A}	0.0208 ^{b,A}

^{a,b,c,A,B,C} Means followed by the same lowercase letter within a column or uppercase letter within a row do not differ at $p > 0.05$ by Tukey's test.

4. Discussion

Euphorbia heterophylla germination was not affected by treatment with aqueous mixtures. However, seedling development was significantly influenced by all tested mixture concentrations. The pattern of changes in respiratory and antioxidant enzyme activities revealed that the production of reactive oxygen species (ROS) started early in *E. heterophylla* development, as assessed during the 4-day incubation period. Root respiration was predominantly cyanide sensitive, suggesting activation of the cytochrome oxidase pathway.

In the presence *U. ruziziensis* aqueous mixture, mitochondria managed to produce substantial amounts of ROS, despite the reduction in mitochondrial respiration and oxygen consumption. This hypothesis was corroborated by the increase in CAT and POD activities. Similar results were observed by Coelho-Pergo et al. [30], who reported that *U. ruziziensis* aqueous extract inhibited mitochondrial respiration and increased CAT activity in *Bidens pilosa* roots after only 4 days of incubation. This result shows that inhibition of mitochondrial respiration induces oxidative stress, as ATP production is reduced. ATP is essential for seedling growth, because, in the initial phase of development, seedlings cannot yet rely on photosynthesis for energy production. Another problem with ATP reduction is that the plant's repair system, that is, the machinery of antioxidant enzymes such as CAT and POD, need this energy source to be produced on a large scale due to the production of ROS. Thus, much of the ATP that is still being produced for the plant goes to the production metabolism of antioxidant enzymes, further hampering energy expenditure with the development of the plant, as seen in this study.

According to Wang et al. [40], mitochondria are key targets for cell death induction. When a molecule binds to mitochondrial membrane receptors, it can directly affect mitochondrial activity and induce fragmentation and mitophagy. These processes occur from depolarization of the mitochondrial membrane, increasing ROS generation and, consequently, the activity of antioxidant enzymes such as superoxide dismutase and CAT.

Foletto et al. [41], demonstrated that water-soluble compounds of *U. ruziziensis* were phytotoxic to *Ipomoea triloba*, disturbing root respiration and lipid peroxidation. *U. ruziziensis* extracts contain compounds such as protodioscin and triterpenoid saponins, which are believed to act against

weeds [17]. Protodioscin [42], is a bidesmosidic saponin formed by a hydrophobic furostanol unit and two sugar units. The compound is easily soluble in water and, therefore, easily absorbed by the plant root. Roots were greatly affected by *U. ruziziensis* extract-mixture in this study. It is probable that protodioscin disturbed the mitochondrial membrane, decreasing respiration via cytochrome oxidase and consequently increasing ROS generation.

Sorghum bicolor root mixture had a similar effect as *U. ruziziensis* mixture on the weed, with the exception that the former did not increase CAT activity. Therefore, it is likely that the mode of action of *S. bicolor* root mixture was similar to that of *U. ruziziensis*.

The respiratory activity of primary roots was probably altered via cytochrome oxidase in the presence of *S. bicolor* stems mixture. However, the increase in KCN-insensitive respiration suggests that the consumption of non-mitochondrial oxygen was stimulated by activation of transient oxidases not linked to the mitochondrial electron chain, including NAD(P)H oxidase, amine oxidase, polyphenol oxidase, oxalate oxidase, peroxidase, and lipoxygenase [43]. These enzymes contribute to KCN-insensitive respiration, which accounted for only 37% of the total respiratory activity in the control. Thus, the 17% increase in non-mitochondrial respiration caused by the mixture is a reflection of the increase in ROS production and CAT and POD activities. Detoxification of hydrogen peroxide is an essential aspect of the response of plants to a variety of stressors [44]. Among other enzymes, class III peroxidases carry out peroxide detoxification. POD isoenzymes may respond differently to the effects of stress.

According to Murimwa et al. [45], aqueous stem extracts of sorghum increased sesame, black jack, and goose grass phenylalanine ammonia-lyase (PAL), POD, and polyphenol oxidase (PPO) activity. Stem extracts induced greater oxidative stress on the test species which in turn stimulated enhanced enzyme activity in the test species. This suggests that stem extracts possess more potent allelochemicals because they contain a variety of potent allelochemicals, including dhurrin, a nonpoisonous glycoside which is hydrolysed to form hydrogen cyanide (HCN), and other phenolic compounds responsible for short-term allelopathic effects.

Zucareli et al. [31], found that *S. bicolor* aqueous extract exerted a dose-dependent inhibitory effect on cabbage germination and initial growth. Extracts were obtained from pre-flowering plants (60 days after emergence), as also performed in the present study. The allelopathic activity of sorghum crops is mainly due to the action of cyanogenic glycosides, tannins, flavonoids, ferulic acid, syringic acid, and vanillic acid [46]. The major allelochemical was found to be the quinone sorgoleone [47]. The compound inhibits the growth of both roots and shoots in various crop and weed species [24,48].

Sorgoleone, together with juglone and flavonoids, greatly affects mitochondrial activity [49–51] when used at a concentration of 20 to 1000 μ M. Phenolic acids are not as active [52]. According to Guenzi et al. [53], decomposed sorghum residues contain substantial amounts of ferulic acid, *p*-coumaric acid, vanillic acid, ferulic acid, and hydroxybenzoic acid. These allelochemicals stimulate ROS production and therefore contribute to the increase in soluble peroxidases. Phenolic acids react directly with sulfhydryl groups that are fundamental to ATPase activity. These compounds induce the activity of oxidative enzymes such as peroxidases and increase free radical production by promoting lipid peroxidation and altering membrane permeability [54]. Simultaneously, lignin synthesis in the cell wall is activated. Lignification is putatively associated with increased permeability. Such associated effects contribute to the reduction of root and plant growth [55]. However, the causal sequence of these events remains unknown.

Aqueous mixtures of *S. bicolor* roots and stems directly interfered with the mitochondrial respiratory chain, that is, in seedling respiration, affecting *E. heterophylla* metabolism. Such effects triggered an intense oxidative response, leading to oxidative stress, activation of peroxidases, and, consequently, inhibition of plant development.

According to the literature studied, it is quite clear that each allelochemical present in each aqueous mixture used in this work may present a mechanism of action within the plant. In this study, however, regardless of this, the mode of action of the three mixtures tested was very similar, as it

reduced mitochondrial respiration and increased the main antioxidant enzymes, generating oxidative stress in the studied plant.

5. Conclusions

Aqueous mixtures of *U. ruziziensis* and *S. bicolor* played a fundamental role in inhibiting *E. heterophylla* seedling development. Their modes of action were probably associated with the effect of allelochemicals on mitochondrial metabolism. Laboratory experiments showed that the highest effects were obtained by using the 1000 ppm mixture. However, it is necessary to investigate the potential of *U. ruziziensis* and *S. bicolor* extracts in inhibiting weed growth under greenhouse or field conditions as active components might be absorbed, retained, transformed, or degraded in soil, thus affecting bioactivity. We conclude that aqueous mixtures of *U. ruziziensis* and *S. bicolor* have phytotoxic effects against *E. heterophylla* and we recommend that they are further evaluated as control agents.

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