Chemical Characterization and Phytotoxic Effects of the Aerial Parts of Ruzigrass (Urochloa ruziziensis)

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Studies of the phytotoxic effects between plants can be a crucial tool in the discovery of innovative compounds with herbicide potential. In this sense, we can highlight ruzigrass (Urochloa ruziziensis), which is traditionally used in the crop rotation system in order to reduce weed emergence. The aim of this work was to characterize the secondary metabolites of ruzigrass and to evaluate its phytotoxic effects. In total, eight compounds were isolated: friedelin, oleanolic acid, α-amyrin, 1-dehydrodiosgenone, sitosterol and stigmasterol glycosides, tricin and p-coumaric acid. Phytotoxic effects of the 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. Chemometric analysis discriminated the weed species into three groups, and B. pilosa was the most affected by fractions of ruzigrass. The phytotoxic activities of 1-dehydrodiosgenone, tricin, and p-coumaric acid are also reported, and p-coumaric acid and 1-dehydrodiosgenone were active against B. pilosa.

Keywords: Bidens pilosa, Euphorbia heterophylla, Ipomoea grandifolia, chemometric analysis, Poaceae, biological activity, phytotoxicity.

Introduction

The Urochloa genus consists of herbaceous, perennial or annual, erect or decumbent plants and belongs to the grass family (Poaceae). This genus, originally from Africa, comprises approximately 100 species. It grows in a wide range of ecosystems from semi-deserts to swamps. Urochloa species are cultivated throughout tropical regions such as Africa, Asia, Australia, and South America as fodder crops, because of its good nutritional quality combined with high seed production, high stress tolerance, high dry biomass production, easy cultivation, good adaptation to different soils for year-round growth, resistance to drought and low maintenance costs. In Brazil, Urochloa species occupy around 100 million hectares of grazing area. Although some species of this genus are used as animal feed, they contain steroidal saponins, which are involved in outbreaks of hepato-genous photosensitization in animals.

Urochloa ruziziensis (R.GERM. & C.M.EVRARD) CRINS (syn. Brachiaria ruziziensis), native to the Ruzi Valley in Zaire (Congo) and Burundi, is commonly known as ruzigrass or Congo grass. This species is widely distributed in...
tropical countries and grows in various types of soils, but it thrives in well-drained soils with good fertility.\textsuperscript{[7,10]} Ruzigrass is cultivated for both animal feed\textsuperscript{[11]} and straw supplies for no-tillage systems.\textsuperscript{[12]} It has increasingly been used as a cover crop in many grain-producing areas of Brazil. It is either intercropped with corn or grown as a single crop in rotation with soybeans\textsuperscript{[13]} because of its highly efficiency in producing biomass, which results in high levels of P and K recovery in soils and nutrient recycling.\textsuperscript{[7,10]} This species also stands out for its adaptability, aggressiveness, and drought-resistance along with its vigorous growth and high tolerance to acidic soils.\textsuperscript{[10]} In addition, some studies have shown that this species reduces weed emergence in fields.\textsuperscript{[11,14–16]}

The aerial parts of ruzigrass were previously screened for their phytotoxic activity against weed species in a greenhouse test, which revealed an inhibitory action on the emergence of the weed species \textit{Euphorbia heterophylla} and \textit{Bidens pilosa}. This effect was observed only when the pots were superficially irrigated, i.e., when water percolated through the straws, suggesting an effect of the secondary metabolites present in the ruzigrass straw in this process of inhibition.\textsuperscript{[12]}

Besides benefits related to water and soil conservation, mulching from ruzigrass may be part of non-chemical strategies for integrated weed control. Thus, identification of the natural compounds in ruzigrass with toxic activity on weeds can boost its use in no-tillage agricultural environments, minimize the use of synthetic herbicide, and reduce environmental contamination and the selection pressure of herbicide-resistant weed biotypes.\textsuperscript{[17]}

Continuing our research on the chemical investigation of species used as a cover crop,\textsuperscript{[18]} and based on the limitation of studies focused on the chemistry of ruzigrass, the present work was planned (a) to isolate and identify the metabolites present in different fractions obtained from the crude methanolic extract of the aerial parts of ruzigrass; (b) to evaluate the activity of fractions, the crude methanolic extract, and some identified compounds on the germination and initial growth of weed species, and (c) to compare the sensitivity of three weed species (\textit{Bidens pilosa} L., \textit{Euphorbia heterophylla} L.\textsuperscript{[20]} and \textit{Ipomoea grandifolia} L.\textsuperscript{[19]} that are commonly found in soybean plantations by using the chemometric data analysis.

### Results and Discussion

The phytotoxic activity of the crude methanolic extract (CME) and hexane (HEXF), dichloromethane (DCMF), and ethyl acetate (EAF) fractions of ruzigrass aerial parts were evaluated on germination, root length, stem length, total length, fresh biomass, and dry biomass of three weed species, \textit{B. pilosa}, \textit{E. heterophylla} and \textit{I. grandifolia}. Results were evaluated by ANOVA with Duncan’s multiple range test ($p \leq 0.05$) and by chemometric data analysis (PCA). The most significant results obtained by ANOVA analysis are described below.

The most sensitive weed species was \textit{B. pilosa}, and it was especially affected by HEXF and DCMF fractions. The germination rate of \textit{B. pilosa} was inhibited only by the DCMF fraction (Figure 1A). However, the HEXF fraction affected all other parameters. It reduced the stem length by 42.84\% (Figure 1C) and seedling dry biomass by 51.40\% (Figure 1F) (Table 1).

\textit{E. heterophylla} was the second most sensitive weed species, and although no fraction inhibited the germination rate (Figure 2A), the total length was affected by all fractions (Figure 2D). The most active fraction against this weed species was the EAF fraction, which inhibited the root length (Figure 2B) and total length (Figure 2D) by 38.53\% and 29.33\%, respectively. This fraction inhibited also the seedling fresh biomass by 16.06\% (Figure 2E) and dry biomass by 25.62\% (Figure 2F) (Table 2).

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Table 1. Results of the phytotoxic effects of CME and HEXF, DCMF, and EAF fractions of ruzigrass on the germination rate, root length, stem length, total length, seedling fresh biomass and seedling dry biomass of \textit{B. pilosa}. Values obtained from seedlings treated with each fraction or crude methanolic extract differing statistically from control seedlings were identified by ANOVA with Duncan’s multiple range test ($p \leq 0.05$). Inhibition is expressed as negative results and stimulation as positive results.
Finally, *I. grandifolia* was the least affected weed species, only the crude methanolic extract was active, and it inhibited all parameters. The germination rate was affected by 15.72% (*Figure 3A*), and the total length (*Figure 3D*), root length and stem length (*Figure 3B and 3C*) were inhibited by approximately 30% (*Table 3*).

Results were also analyzed using PCA, which is a mathematical approach that is used to summarize the
information contained in a multivariate dataset by reducing the dimensionality of the data without losing important information and to recognize possible differences or similarities among the samples. Chemo-
metric analysis of HEXF, DCMF, EAF fractions and CME of ruzigrass was performed using 12 means from 59 measurements. Results obtained from the PCA showed the first component PC1 explained 83.67\% and PC2

Figure 2. Phytotoxic effects of CME and HEXF, DCMF, and EAF fractions of ruzigrass on the germination rate (A), root length (B), stem length (C), total length (D), seedling fresh biomass (E) and seedling dry biomass (F) of E. heterophylla. Seeds were incubated for 96 h in the presence of methanol (control) or each treatment tested (500 \(\mu\)g mL\(^{-1}\)). Each data point is the mean ± SEM, \(n = 5\). Significant differences between means were identified by ANOVA with Duncan’s multiple range test (\(p \leq 0.05\)). * = values obtained from seedlings treated with each fraction differing statistically from control seedlings.
explained 13.06% of the variance. Thus, the cumulative results of the first two principal components (PCs) accounted for almost 97% of the total variance in the original data. Figure 4 shows the principal component scores and loadings plot using PC1 and PC2 which were able to discriminate the weed species into three different groups, and the principal component loadings plot indicates the important variables contributing to the PCs. In addition, it shows that each weed species was affected differently by fractions and the crude methanolic extract of ruzigrass. Group I is located in the negative region of scores of PC1 and corresponds to the effects on *B. pilosa*. Group II is located in the positive region of PC2 and consists of the effects on *E. heterophylla* and the phytotoxic activity of the DCMF fraction on *I. grandifolia*. Group III is located in the positive region of PC1 and represents the effects on *I. grandifolia* (Figure 4).

According to the loadings plot, the germination rate, stem length, total length, and fresh biomass were the variables that most influenced discrimination of the group, and dry biomass was the parameter that least influenced this discrimination. Discrimination using the influence exhibited by the assayed parameters is inversely proportional to the phytotoxic activity. Thus, if a parameter influenced a group, then, this parameter was not inhibited by CME or any fractions of ruzigrass.

Group I was not influenced by any parameters, so the CME and HEXF, DCMF, EAF fractions of ruzigrass exhibited phytotoxic activity on *B. pilosa* and inhibited all parameters. Group II was discriminated mainly by germination rate, which means that the CME and HEXF, DCMF, EAF fractions of ruzigrass inhibited all of the other parameters of *E. heterophylla*, whereas *I. grandifolia* is located in Group III, which was influenced by almost all parameters, it was shown that this weed species was the least sensitive weed species.

HCA is another unsupervised clustering method used to establish the relationships among similar groups of samples. HCA results (Figure 5) revealed that regarding the activities of the CME and HEXF, DCMF, and EAF fractions of ruzigrass, *E. heterophylla* exhibits a closer relationship with *I. grandifolia* than with *B. pilosa*. This result was consistent with the fact that these two weed species were less sensitive to fractions and the crude methanolic extract of ruzigrass when compared to *B. pilosa*. HCA results were also consistent with the results from the PCA, which separated the three weed species into three groups.

A significant difference in weed sensitivity to ruzigrass extracts was observed. This is not uncommon, because many factors can contribute to the effectiveness of an active compound on a receptor plant. These factors may be related to the physicochemical properties of chemical constituents of ex-
tracts, and the differences in the anatomical, physiological and biochemical characteristics of each species. As well, there are reports that discuss the susceptibility of weed species to plant extracts and their relation to seed size. Souza Filho et al. evaluated the phytotoxic effects of Calopogonium mucunoides on the weeds Mimosa pudica, Urena lobata, Senna obtusifolia, and Senna occidentalis and
Figure 4. PCA scores and loadings plot for the phytotoxic activity of CME and HEXF, DCMF, and EAF fractions of ruzigrass tested on three weed species, B. pilosa, E. heterophylla and I. grandifolia.

Figure 5. Dendrogram of HCA using Ward’s minimum variance method of the three weed species tested for the phytotoxic activity (I. grandifolia – blue, E. heterophylla – green, B. pilosa - red). In HCA, clustering of samples is based on their similarity.
revealed that the intensity of phytotoxic effects varied negatively with the increase of seed size. Generally, small-seeded species have less nutritional reserve mass to support the initial phase of the seedling deployment than large-seeded species. In addition, small-seeded species have a larger root length per unit root mass, which contributes to a relatively higher absorptive surface area through which fractions or compounds are absorbed.\(^{[26]}\) In agreement with these assumptions, \(B.\ pilosa\), a small-seeded weed species, was the most sensitive to crude methanolic extract or fractions of ruzigrass, and \(I.\ grandifolia\), a large-seeded weed species, was less sensitive.

Less absorption and translocation of the active compounds, or a faster metabolic degradation, may also contribute to different sensitivities of the weed species.\(^{[27]}\) In this work, the three weeds tested are dicotyledons and belong to three different families, Convolvulaceae (\(I.\ grandifolia\)), Asteraceae (\(B.\ pilosa\)) and Euphorbiaceae (\(E.\ heterophylla\)). Seeds of the Convolvulaceae family exhibit, in general, physical dormancy, a waterproof seed coating.\(^{[28]}\) This rigid tegument provides an important barrier for the embryo against external influences such as the absorption of compounds by the seeds.\(^{[29]}\) Although in the present work the seeds were chemically treated to break dormancy, it is possible that a lower absorption rate of the crude methanolic extract or fractions of ruzigrass contributed to lower sensitivity in comparison with the other weeds.

Our work demonstrated that root length was the parameter of seedling development more affected by the crude methanolic extract and fractions than the stem length in all tested weeds. Inhibition of the root and stem lengths may be related to modifications of essential processes such as cell division, phytohormones signaling, membrane permeability, enzymatic activity, hydric stress, and changes in DNA synthesis, among others.\(^{[30–32]}\) Moreover, primary roots remain in direct contact with the crude methanolic extract or fractions, which can damage this organ more easily than primary stems.\(^{[23]}\)

The phytochemical study of HEXF, DCMF, and EAF fractions obtained from aerial parts of ruzigrass yielded eight compounds, and the structures of the compounds were established by comparison of their spectroscopic 1D and 2D NMR data with those reported in the literature. The HEXF fraction afforded three triterpenes, friedelin (1),\(^{[33]}\) oleanolic acid (2)\(^{[34]}\) and \(\alpha\)-amyrin (3),\(^{[35]}\) and one steroidal sapogenin, 1-dehydrodiosgenone (4),\(^{[36]}\) Friedelin (1), two glycoside phytosterols, sitosterol 3-O-glucoside (5)\(^{[37]}\) and stigmasterol 3-O-glucoside (6),\(^{[37]}\) and one flavone, tricin (7), were obtained from the DMF fraction.\(^{[38]}\) The EAF fraction afforded one phenylpropanoid, \(p\)-coumaric acid (8),\(^{[39]}\) and tricin (7). These compounds are being described for the first time in ruzigrass, with exception of \(p\)-coumaric acid (Supporting Information).\(^{[40]}\)

The compounds 1-dehydrodiosgenone (4), tricin (7), and \(p\)-coumaric acid (8), isolated during the phytochemical investigation of ruzigrass aerial parts, were assayed to verify the influence of these compounds on the germination rate, root length, stem length, total length, seedling fresh biomass and seedling dry biomass of \(B.\ pilosa\), because this species was the most affected in the experiments with the crude methanolic extract and fractions of ruzigrass. Limited by sample amounts, we were only able to evaluate the phytotoxic activity of these three compounds. However, the other compounds are usually associated with phytotoxic activity.\(^{[41–46]}\)

Figure 6 shows the phytotoxic effects of selected compounds on the germination rate (A), initial seedling growth (B, C, and D), and seedling fresh (E) and dry biomass (F) of \(B.\ pilosa\). For comparative purposes, all fractions were assayed at 250 \(\mu\)g mL\(^{-1}\). Results showed that \(p\)-coumaric acid (8) inhibited approximately 33.50% of root length (Figure 6B), stem length (Figure 6C), and total length (Figure 6D), when compared with the untreated controls. In addition, \(p\)-coumaric acid reduced seedling fresh biomass by 44.65% (Figure 6E). Phenylpropanoids are already known as allelochemicals, and they can affect various physiological processes in other plants.\(^{[47]}\) For example, \(p\)-coumaric acid was tested against the weed species \(S.\ occidentalis\), \(S.\ obtusifolia\), and \(M.\ pudica\) and results showed that it inhibited the germination rate of seeds and root development of \(S.\ occidentalis\) and \(M.\ Pudica\).\(^{[39]}\) Tricin (7) did not show any phytotoxic activity at the concentration of 250 \(\mu\)g mL\(^{-1}\), although there are reports of tricin inhibiting the growth of the weed species \(Echinochloa\ crus-galli\), \(Cyperus\ difformis\) and \(Lepidium\ sativum\).\(^{[48,49]}\) The steroidal sapogenin, 1-dehydrodiosgenone (4), affected only one parameter, inhibiting stem length by 22.76% (Figure 6C). However, it should be emphasized that it is the first time that the phytotoxic activity of this compound on a weed has been reported.

Conclusions

In the present work, we investigated the chemical composition as well as the phytotoxic effects of the
Figure 6. Phytotoxic effects of 1-dehydrodiogenone, tricin, and p-coumaric acid isolated from ruzigrass on the germination rate (A), root length (B), stem length (C), total length (D), seedling fresh biomass (E) and seedling dry biomass (F) of *B. pilosa*. Seeds were incubated for 120 h in the presence of methanol (control) or each compound tested (250 μg mL\(^{-1}\)). Each data point is the mean ± SEM, \(n = 5\). Significant differences between means were identified by ANOVA with Duncan’s multiple range test (\(p \leq 0.05\)). * = values obtained from seedlings treated with each fraction differing statistically from control seedlings.
crude methanolic extract and fractions of ruzigrass. All of isolated compounds were described for the first time in this species, with exception of p-coumaric acid. These results contribute to the phytochemical knowledge of the *Urochloa* genus. Our work revealed that aerial parts of ruzigrass contain chemical compounds that are able to suppress the emergence of weed species with different effectiveness as revealed by chemometric analysis. Considering that populations of weeds in this study have already been selected as resistant to important classes of herbicides such as EPSPS, ALS, PS I, PS II, and PROTOX inhibitors, ruzigrass may represent a very important contribution in weed management in tropical agricultural environments.

**Experimental Section**

**General Experimental Procedure and Materials**

Nuclear Magnetic Resonance (NMR) experiments were recorded on a VARIAN Mercury Plus spectrometer (Palo Alto, CA, USA) operating at 300 MHz and 75.5 MHz or on a Bruker AVANCE III HD spectrometer (Karlsruhe, Germany) operating at 500 MHz and 125 MHz using CDCl$_3$ and CD$_3$OD as solvents. Chemical shifts are given in ppm, and solvent signals were used as references for $^1$H and $^{13}$C signals. Chromatography columns were performed using silica gel 60 (0.063 – 0.200 mm, 70 – 230 mesh, Merck, Darmstadt, Germany) or Sephadex LH-20 (i.d. 3.5 cm × 34.5 cm) using mixtures of MeOH/H$_2$O (20:80, 40:60, 60:40, 80:20 and 100:0; v/v) as eluents. This procedure was repeated twice to afford five fractions: DCMF-1 (6.1 g), DCMF-2 (1.4 g), DCMF-3 (2.6 g), DCMF-4 (2.7 g), and DCMF-5 (2.1 g). Part of the DCMF-1 fraction (0.4 g) was separated by silica gel 60 CC (i.d. 2.0 cm × 33.5 cm) eluted with mixtures of hexane/AcOEt and AcOEt/MeOH at different increasing polarities (0 to 100%; v/v) to give subfractions DCMF1-1 to DCMF1-26. Compound 1 (6.7 mg) was reisolated from subfraction DCMF1-1 after CC (i.d. 1.0 cm × 21.0 cm) in silica gel using a gradient of hexane to AcOEt as eluent. The mobile phase was MeOH 100 % as an isocratic eluent, to give subfractions DCMF1-1 to DCMF1-26. Compound 1 (6.7 mg) was reisolated from subfraction DCMF1-1 after CC (i.d. 1.0 cm × 21.0 cm) in silica gel using a gradient of hexane to AcOEt as the mobile phase, and the subfraction DCMF1-14 yielded a mixture of compounds 5 and 6 (3.1 mg). Subfraction DCMF-2 (1.3 g) was subjected to a Sephadex LH-20 CC (i.d. 3.5 cm × 34.5 cm) using MeOH 100 % as an isocratic eluent, to give subfractions DCMF-2-1 to DCMF-2-31. Subfraction DCMF-2-27 yielded compound 7 (8.5 mg).

Part of the EAF fraction (8.0 g) was subjected to a Sephadex LH-20 CC (i.d. 3.5 cm × 34.5 cm) using mixtures of MeOH/H$_2$O (20:80, 40:60, 60:40, 80:20 and
100:0; v/v) as eluent to afford five subfractions: EAF-1 (5.2 g), EAF-2 (0.9 g), EAF-3 (0.6 g), EAF-4 (0.2 g), and EAF-5 (0.1 g). Part of the EAF-2 fraction (200 mg) was subjected to a Sephadex LH-20 CC (i.d. 2.5 cm × 20.0 cm) eluting with MeOH/H₂O 50% and MeOH 100% to give subfractions EAF-2-1 to EAF-2-26. Compound 7 (15.1 mg) was reisolated from subfraction EAF-2-23. Subfraction EAF-2-14 (65.0 mg) was submitted to a Sephadex LH-20 CC (i.d. 1.0 cm × 20.5 cm) using CHCl₃/MeOH 50% as an isocratic eluent, to give subfractions EAF-2-14-1 to EAF-2-14-12. Further purification of EAF-2-14-11 (35.0 mg) using Sephadex LH-20 CC (i.d. 2.5 cm × 19.5 cm) and 100% MeOH isocratic as the mobile phase, resulted in the isolation of compound 8 (14.9 mg).

Evaluation of Phytotoxic Activity of Crude Methanolic Extract and Fractions of Ruzigrass

The phytotoxic activity of CME and HEXF, DCMF, and EAF fractions obtained from the aerial parts of ruzigrass were evaluated against three weed species, B. pilosa, E. heterophylla and I. grandifolia. The parameters selected to assess the activity on weed development were the germination rate, root length, stem length, total length, seedling fresh biomass, and seedling dry biomass. B. pilosa seeds were soaked in 1% sodium hypochlorite for 5 min and then, washed with distilled water. E. heterophylla seeds were only washed with distilled water. I. grandifolia seeds were soaked in sulfuric acid for 45 min and then, washed with distilled water. Crude methanolic extract and fractions of ruzigrass (25 mg of each treatment) were resuspended in 100% methanol and placed on double sheets of germination paper in plastic germination boxes (110 × 110 mm). The final concentration of CME and HEXF, DCMF, and EAF fractions of ruzigrass were used, but with some modifications. The final concentration was 250 μg mL⁻¹ (12.5 mg of each compound) and seeds that had germinated after 120 h were selected for growth tests. Purity of the tested compounds was determined by ¹H-NMR.

Statistical Analysis

Multivariate statistical analysis was performed on the free statistical software R 3.4.1 version (RStudio 1.0.153, https://www.r-project.org) using Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). Both methods were implemented on ggfortify and factoextra packages for R. Data obtained from the phytotoxic activity using the fractions and the crude methanolic extract of ruzigrass were expressed as means. Means were preprocessed by autoscaling (mean centering and scaling to unit variance) and normalizing using a data matrix of 12 means and 6 variables. In addition, HCA was carried out using Ward’s minimum distance variance as the similarity measurement. The phytotoxic activity of the crude methanolic extract, fractions, and pure compounds of ruzigrass were expressed as means ± standard errors (SEM) of independent preparations. The data were submitted to one-way variance analyses (ANOVA), and significant differences between the means were identified by Duncan’s test using p ≤ 0.05 as the minimum criterion of significance. Statistical analyses were performed using the Statistica™ software package.
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Author Contribution Statement

This work was conducted by B. P. M. Plant material was cultivated and harvested by R. S. O. Jr. Isolation, identification and evaluation of phytotoxic activity were furnished by B. P. M., G. C. M., L. L. R., A. A. S. Chemometric analysis was carried out and discussed by B. P. M. The manuscript was prepared by B. P. M., E. L. I.-I., R. S. O. Jr., M. H. S., and D. C. B. All of the authors revised the manuscript.

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