Research Article

Received: 20 February 2017 Accepted article published: 26 March 2017 Published online in Wiley Online Library: 24 May 2017

(wileyonlinelibrary.com) DOI 10.1002/ps.4578

Chemical evidence for the effect of *Urochloa ruziziensis* on glyphosate-resistant soybeans

Mariluce Nepomuceno,[a] Nuria Chinchilla,[b] Rosa M Varela,[b] José MG Molinillo,[b] Rodney Lacret,[b] Pedro LCA Alves[a] and Francisco A Macias[b*]

Abstract

BACKGROUND: Soybean (*Glycine max*) is an important oleaginous legume that has been cultivated in new areas in Brazil, including pastures. Problems of reduced production yields have been reported by soybean growers when the crop is sown immediately after desiccation of pastures of *Urochloa* spp. using glyphosate. The objective of this work was to extract, isolate and identify the major chemicals from *U. ruziziensis* that have phytotoxic activity and to evaluate the possible relation between this effect and reduced soybean yield.

RESULTS: *U. ruziziensis* plants at the flowering stage were desiccated using glyphosate at 1.44 kg ha^{-1}. The plants were collected between five and ten days after treatment. Extracts of dried and ground shoots were obtained by sequential extraction with hexane, dichloromethane and methanol. The results of wheat coleoptile bioassays indicated that the methanol extract was more inhibitory than the dichloromethane extract regardless of glyphosate application.

CONCLUSION: Protodioscin, a steroidal saponin, was isolated from the extract as the major component and the activities of this compound were in good agreement with those found for the extract. The release of this compound into the soil is a plausible explanation for the decrease in production observed in transgenic soybean crop after desiccation of *U. ruziziensis*.

Keywords: soybean; glyphosate; protodioscin; *Urochloa ruziziensis*; allelopathy

1 INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is an important oleaginous legume that is planted widely in Brazil and has been cultivated in new areas, including pastures. The cultivation of this plant has been one of the main alternatives for the recovery and restoration of degraded pastures. Problems of reduced production yields have been reported by soybean growers when the crop is sown immediately after desiccation of pastures of *Urochloa* spp. using glyphosate.[1]

In Brazil, as in others countries, the adoption of planting systems is aimed at increasing soil conservation, which in turn is associated with good yields.[2] The residues produced by commercial crops are generally insufficient for good ground cover.[3] Therefore, it is necessary to introduce plants that are capable of producing a large amount of biomass, with an initial rapid development and a short cycle. In addition, their residues should not decompose quickly so that the soil remains covered for as long as possible.

The species of the genus *Urochloa* (syn. *Brachiaria*, Poaceae) are important herbages of tropical regions like Africa, Asia, Australia and South America. It is estimated that Brazil has around 100 million hectares of cultivated grass. In the Cerrado alone these occupy an area of 54.1 million hectares, or 26.4% of the biome,[4] and these mostly contain plants of the genus *Urochloa*. In general, this genus is considered to be the preferred choice for the formation of plant cover for farming due to the good production of dry material (over 15 t ha^{-1}). Additionally, this genus has higher concentrations of lignin in the plant constitution, which give it a high C/N ratio. This feature is related to the decomposition time of the plant material,[5] which may persist for more than six months on the soil surface.[6]

In particular, the species *Urochloa ruziziensis* Germ. & Evrard (syn. *Brachiaria ruziziensis*) is a plant that can grow in various soils, from sandy to clayey, and it develops best in well-drained soils with good fertility. The biomass production of these plants results in high levels of P and K present in soil.[7,8] *U. ruziziensis* has faster decomposition and lower half-life values than other plants and this in turn leads to nutrient recycling and higher maize yields.[9]

Nunes *et al.* pointed out that the use of straw from *Urochloa* for ground cover requires information about the ideal drying period between planting and harvest, so that a negative impact does not occur on successive crops.[5] There are several reports of symptoms of yellowing and a reduction in the growth and productivity of conventional soybean plants[10–12] when they are used in areas

* *Correspondence to: FA Macias, Allelopathy Group, Department of Organic Chemistry, Institute of Biomolecules (INBIO), Campus de Excelencia Internacional Agroalimentario (ceiA3), University of Cádiz, C/ Avda República Saharaui 7, 11510 Puerto Real, Cádiz, Spain. E-mail: famacias@uca.es*

a *Department of Applied Biology to Agropecuary, São Paulo State University, Jaboticabal, Brazil*

b *Department of Organic Chemistry, Institute of Biomolecules (INBIO), University of Cádiz, Cádiz, Spain*

that have undergone pasture tillage with *Urochloa*.[13,14] This effect is more obvious and pronounced immediately after desiccation of plant cover.[14] One of the most widely used herbicides for the desiccation of cover crops is glyphosate.

The objective of the study reported here was to explain the adverse effects observed on soybean crops in areas where *U. ruziziensis* had been used as a cover crop. With this aim in mind, plant extracts of *U. ruziziensis* collected at different times after the application of glyphosate were evaluated for phytotoxic effects on transgenic soybean cv. M-SOY 7908 RR. In addition, a bioassay-guided isolation of active compounds present in the extracts was carried out, with the first stage involving an evaluation of the activity of the extracts obtained from the aerial part of *U. ruziziensis* both with and without the application of glyphosate. In the second stage, the main compounds in the aerial part of *U. ruziziensis* were isolated and identified.

2 EXPERIMENTAL METHODS

2.1 General procedures

Infrared spectra (KBr) were recorded with an FT-IR Spectrum 1000 spectrophotometer (PerkinElmer, Waltham, MA, USA). Nuclear magnetic resonance (NMR) spectra were recorded using a Varian INOVA-600 spectrometer equipped with a 5 mm ^{1}H $\{^{15}N-^{31}P\}^{15}$ PFG high-field inverse detection z-gradient probe (Agilent, Palo Alto, CA, USA). ^{1}H NMR (599.78 MHz) and ^{13}C NMR (150.83 MHz) spectra were recorded in MeOH-d_4 at 25 °C. Chemical shifts are given in ppm with respect to the residual solvent signal (3.30 ppm) and ^{13}C signals are referenced to the solvent signal (49.00 ppm). High-resolution mass spectra were obtained with a Synapt G2 UPLC-QTOF ESI mass spectrometer (Waters, Milford, MA, USA).

MPLC separation was carried out using a Büchi 861 apparatus with a 40–63 µm LiChrospher RP-18 column using acetone–H_2O (1:1) as the mobile phase. HPLC was carried out using an HPLC system (Merck-Hitachi, Tokyo, Japan) with refractive index detection. Silica gel (0.060–0.200, 60A) from Acros Organics (Geel, Belgium) and Lichroprep RP 18 (40–63 µm) from Merck (Darmstadt, Germany) were used for column chromatography. HPLC columns used were semipreparative 250 mm × 10 mm i.d., 10 µm Lichrospher 100 RP-18 columns (Merck, Darmstadt, Germany) fitted with a guard column (LiChrospher RP-18, Merck, Darmstadt, Germany) and an analytical 250 mm × 4.5 mm i.d., 5 µm Gemini 110A RP-18 column (Phenomenex, Torrance, CA, USA) with a guard column (SecurityGuard Cartridges Gemini RP-18, Phenomenex, Torrance, CA, USA). Thin layer chromatography (TLC) was performed on TLC silica gel 60 F_{254} aluminium sheets and TLC silica gel 60 RP-18 F_{254S} aluminium sheets from Merck (Darmstadt, Germany).

Ultrasound extractions were performed using an ultrasonic bath (360 W, JP Selecta, Barcelona, Spain) for 15 min.

2.2 Chemicals

n-Hexane, methanol (MeOH), dichloromethane (DCM) and acetone (Hipersolv Chromanorm for HPLC) were obtained from VWR International (Radnor, PA, USA). MagniSolv MeOH-d_4 (minimum deuteration degree of 99.8%) for NMR spectroscopy was from Merck (Darmstadt, Germany).

2.3 Plant material

U. ruziziensis plants were grown in the Faculty of Agricultural Sciences of UNESP (Jaboticabal), in an experimental area belonging to the Department of Applied Biology and Agriculture, in brick cold frames built on the ground, with an area of 1.33 m^2, which made up the plots. The plots were filled with a substrate that was clay-texture typical eutrophic Red Latosoil (Andrioli; Centurion, 1999) with the following chemical properties: pH ($CaCl_2$) of 6.0; MO of 39.00 mg dm^{-3}, 40.0 mg dm^{-3} of P (resin); 5.5, 49.0, 31.0, 22, 85.5 and 107.0 mmol dm^{-3} of K, Ca, Mg, H + Al, SB and T, respectively, and 80% V. In July 2008, approximately 200 *U. ruziziensis* seeds were broadcast sown and superficially incorporated into the soil on six of the plots. Fertilizer was also applied using broadcast application based on soil testing and on the crop's nutrient requirements, with the equivalent of 500 kg ha^{-1} of formula 04-14-08 applied per plot. Topdressing with the equivalent of 150 kg ha^{-1} of nitrogen was applied around 45 days after emergence. When the *Urochloa* plants began to flower, at around 120 days after emergence and when they had reached an approximate height of 0.7 m, the crops from four plots were desiccated using glyphosate herbicide and two plots were left untreated to harvest plant material that had not been treated with herbicide (controls). For desiccation, 1.44 kg ha^{-1} of glyphosate was applied using a backpack precision sprayer equipped with sprinkle bars and two flat jet spray nozzles (TT 110.02), 0.5 m apart, with a spray solution consumption rate of 200 L ha^{-1} at a constant pressure of 2.24 kgf cm^{-2}. Around 5 to 10 days after desiccation, the aerial parts of the plants were collected from each plot by hand using pruning shears, and the material collected was dried in the shade at room temperature in order to obtain consistent dry matter (similar conditions to the field). The same method was used for the control, which had not been treated with herbicide, where the plants, which were still green, were cut by hand and then dried in the shade.

2.4 Sample extraction and isolation

2.4.1 Extracts

A mixture of dried material (10 g) and 30 mL of *n*-hexane at room temperature was placed in an ultrasonic bath during 15 min at room temperature in order to de-fat the material. The plant residue was re-extracted using two different solvents. DCM and MeOH were used to extract de-fatted material (3 × 30 mL of solvent) using an ultrasonic bath during 15 min at room temperature. The supernatant was filtered under vacuum and the solvent was removed under reduced pressure (IKA model RV8 basic). In total six extracts were obtained: three in MeOH and three in DCM for material without treatment with glyphosate (M-NT, D-NT), material collected five days after treatment (M-5AT, D-5AT) and ten days after treatment (M-10AT, D-10AT). All of the extracts were bioassayed with etiolated wheat coleoptiles. The MeOH extracts were the most active.

In a second step, bearing in mind the data obtained in the bioassays, a second extraction was carried out on 0.3 kg of plant material using the procedure described above, but in this case only under two of the sets of conditions tested: i.e. not treated with glyphosate (M-NT) and ten days after treatment (M-10AT). The samples corresponding to five days after treatment (M-5AT) were not used because of the similarity between the results of bioassays with the material obtained five and ten days after treatment.

2.4.2 Isolation and purification

The best activity results were obtained with extracts in MeOH and, as a consequence, only those extracts were selected for fractionation. The solvent was removed and 12 g of MeOH extracts M-NT and M-10AT were purified by VLC on LiChrospher RP-18 and eluted with mixtures of MeOH–H_2O (4:0, 3:1, 2:2, 1:3 and 0:4,

each 2.0 L) to give five fractions (M-NT-1 to M-NT-5 and M-10AT-1 to M-10AT-5). All of the fractions were bioassayed with etiolated wheat coleoptiles. The most active fractions were M-10AT-3 (2:2, 3 g) and M-10AT-4 (3:1, 390 mg).

The fraction M-10AT-3 was subjected to MPLC in portions of 300 mg using a Büchi 861 apparatus with a column filled with 40–63 µm LiChrospher RP-18, using 40% acetone–H_2O as the mobile phase at a flow rate of 2 mL min^{-1}. A total of 12 millilitre fractions were collected and assessed using TLC on RP-18 $F_{254}S$, developed with acetone–H_2O (4:6), then sprayed with oleum reagent and heated at 150 °C. This separation gave five fractions (M-10AT-3a to M-10AT-3e). Fraction M-10AT-3c was purified by HPLC using a semipreparative column (Lichrospher R 100-RP 18–10 µm) using acetone–H_2O to 50% as eluent at a flow rate of 3 mL min^{-1}. Protodioscin (**1**; 1.4 g) was isolated (Fig. 1).

The M-10AT-4 fraction was subjected to a similar isolation process. Firstly, the fraction was subjected to MPLC using 60% acetone–H_2O as mobile phase at a flow rate of 1 mL min^{-1} to give five fractions (M-10AT-4a to M-10AT-4e). The M-10AT-4e fraction was purified by HPLC using the same conditions as described above. Once again **1** was isolated but in this case a mixture of dioscin (**2**) and its epimer at C-25 (**3**) (17.9 mg) was also present.

2.5 Bioassay testing

2.5.1 Coleoptile bioassay

Wheat seeds (*Triticum aestivum* L. cv. Cortex) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 25 ± 1 °C for 4 days.[16] The roots and caryopses were removed from the shoots. The latter were placed in a Van der Weij guillotine and the apical 2 mm was cut off and discarded. The next 4 mm of the coleoptiles was removed and used for bioassays. All manipulations were performed under a green safelight.[17] Crude extracts, fractions or pure compounds were predissolved in dimethyl sulfoxide (0.1%) and diluted in phosphate–citrate buffer containing 2% sucrose[17] at pH = 5.6 to the final bioassay concentrations (0.8, 0.4 and 0.2 mg mL^{-1} for extracts and fractions, and 10^{-3}, 3×10^{-4}, 10^{-4}, 3×10^{-5} and 10^{-5} M for compounds).

Parallel controls were also run. The commercial herbicide Logran®, whose original formulation is a combination of N^2-*tert*-butyl-N^4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine (terbutryn, 59.4%) and 1-[2-(2-chloroethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea (triasulfuron, 0.6%), was used as positive control according to a comparison study reported previously.[18] This reference was used at the same concentrations and under the same conditions as reported previously. Control samples (buffered aqueous solutions with dimethyl sulfoxide and without any test compound) were used for all of the plant species assayed.

Each assay was carried out in duplicate. Five coleoptiles and 2 mL of solution were placed in each test tube (three tubes per dilution) and the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 25 °C in the dark. The coleoptiles were measured by digitalization of their images. Data were statistically analysed using Welch's test.[19] Data are presented as percentage differences from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition.

2.5.2 Phytotoxicity bioassays on soybean seedlings

Soybean seeds of the variety M-SOY 7908 RR were germinated on gerbox plates of 100 cm^2 (30 seeds per plate) in a growth chamber (MEMMERT, model ICP 800) set at 25 °C, with a photoperiod of 12 h/12 h light/dark and wet with 12 mL of distilled water. After four days the seeds were transferred to glass containers (20 cm × 9 cm, 4 pre-germinated seeds per pot) containing 0.1 L of glass perlite. Germination and growth were conducted in aqueous solutions at controlled pH using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid and 1 M NaOH (pH = 6.0). The extracts, fractions or compounds were assayed at given concentrations for each extract or fraction (0.8, 0.4 and 0.2 mg mL^{-1}) and compound (10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5}, 5×10^{-6} and 10^{-6} M). Parallel controls were also run. The herbicide glyphosate was used as positive control and was tested at 10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5}, 5×10^{-6} and 10^{-6} M. Commercial products were tested at concentrations of 10^{-3}, 5×10^{-4} and 10^{-4} M. In all tests, 20 mL of solution was added to each sample and four replicates were used. Bioassays took seven days. After growth, the evaluated parameters (root length RL, shoot length SL, dry weight root DWR and dry weight shoot DWS) were recorded. Data were analysed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition.

2.5.3 Statistical analysis

Data were statistically analysed using Welch's test, with significance fixed at 0.01 and 0.05. Results are represented in bar charts as percentages from the control, as described above.

2.5.4 Calculation of IC_{50}

Data from wheat coleoptile and Petri dish seedling bioassays were fitted to a sigmoidal dose–response model (constant slope), whenever possible, using the GraphPad Prism v.4.00 software package.[20]

3 RESULTS AND DISCUSSION

Problems of reduced production yields in soybean have been reported when the crop is sown immediately after desiccation of pastures of *Urochloa* spp. with glyphosate.[1] It is postulated that the detrimental effect may be due to an increase in the applied dose of the herbicide or the number of applications, which are post-emergence in soybean. This effect could therefore be related to the time of application or to a possible negative interference with soil microorganisms.[21–24] The hypothesis is that the observed adverse effects on crops are due to glyphosate degradative processes within the plant resulting in the formation of aminomethylphosphonic acid, which is a known phytotoxin.[25–27] To test the hypothesis, the phytotoxic effect caused by the herbicide glyphosate on commercial soybean seedling variety M-SOY 7908 RR was evaluated. The results show that glyphosate did not produce significant inhibition of any of the parameters evaluated (Fig. 2) but a slight stimulation thereof. The results of several studies may explain this observation.[28] Morandi found that the colonization of soybean by *Glomus mosseae* increases with the application of glyphosate after emergence of the plants. The author suggests that the positive effect of glyphosate may result in increased production of isoflavones in the roots, which can in turn promote mycorrhizal colonization.[29]

Another hypothesis to explain the production losses in soybean crops is that the decomposition of vegetation cover involves the production and/or release of allelopathic compounds that affect

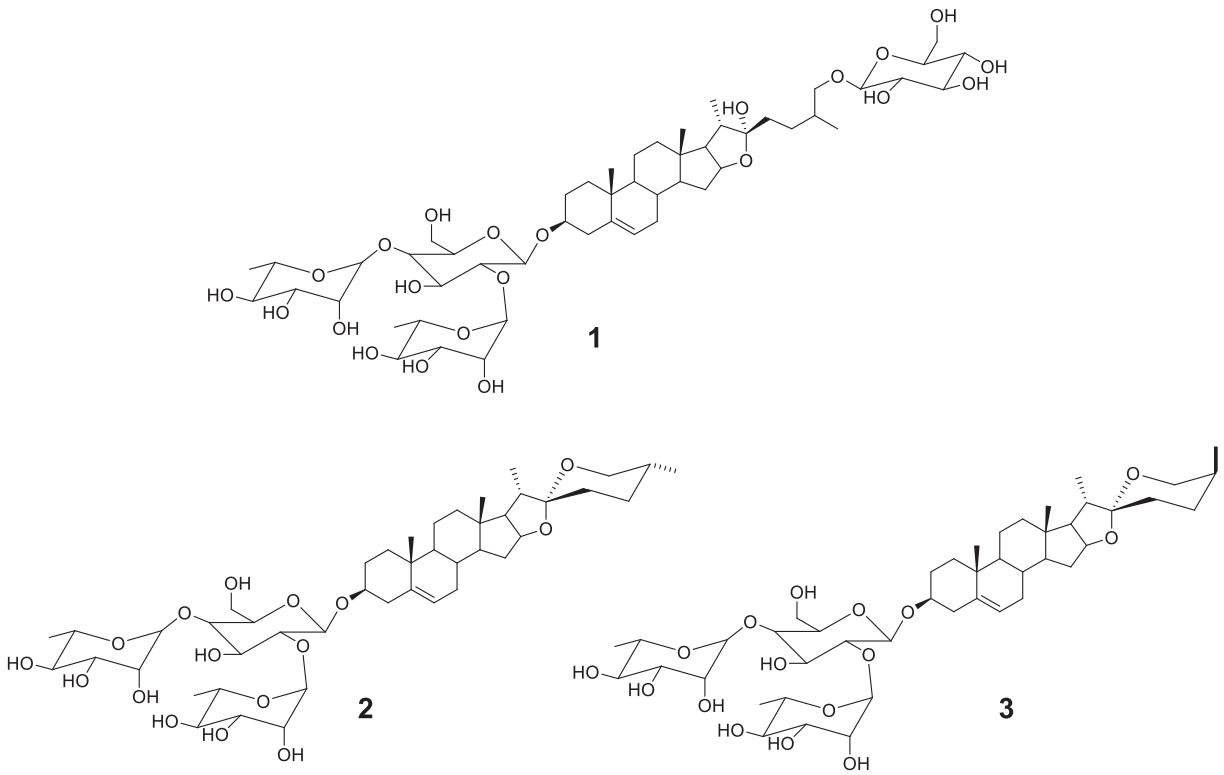

Figure 1. Steroidal saponins isolated from *Urochloa ruziziensis*.

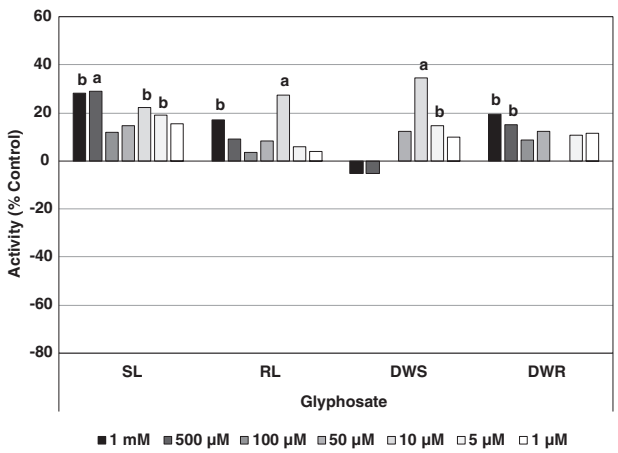

Figure 2. Effects of concentration of glyphosate on transgenic soybean seedlings. The parameters evaluated were root length (RL), shoot length (SL), dry weight root (DWR) and dry weight shoot (DWS).

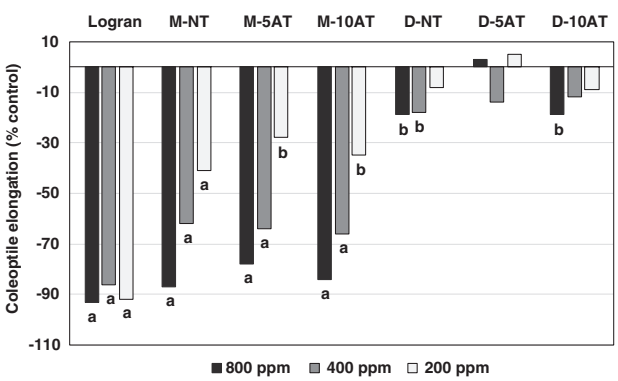

Figure 3. Bioassay with etiolated wheat coleoptiles on the initial extracts.

the growth of weeds[30] but can also damage crops.[31–33] Studies show that plants of the genus *Urochloa* have allelopathic inhibitory activity on seed germination and the development of plants of different species.[31,34] Studies conducted with this genus have shown allelopathic activity in both the seeds and the aerial part, with the aerial part being the main source of chemicals with potential allelopathic activity.[35]

Several studies have shown that during plant-residue decomposition of some allelopathic plants, compounds released include phenolic acids that affect weed germination and seedling development.[36–40] The allelopathic behaviour of *U. ruziziensis* has been related to the presence of *o*-coumaric and aconitic acids.[32,41] To confirm this hypothesis, the bioactivity was tested on soybean seedling variety M-SOY 7908 RR. The compounds did not show significant effect on any of the parameters evaluated. Therefore, the release of these compounds to the soil is not the cause of yield loss observed in soybean crops.

In view of these results, it was decided to carry out a bioassay-guided isolation to identify potential products responsible for the observed inhibitory activity for *U. ruziziensis*. Dried leaves of *U. ruziziensis* were de-fatted with hexane and were then extracted with DCM and MeOH. The extracts were tested in the etiolated wheat coleoptile bioassay.[18] This is a rapid test that is sensitive to a wide range of bioactive substances[42] including plant growth regulators and herbicides. A total of six extracts were assayed, three in MeOH (M-NT, M-5AT, M-10AT) and three in DCM (D-NT, D-5AT, D-10AT), as described in Section 2. The results of the assay are shown in Fig. 3.

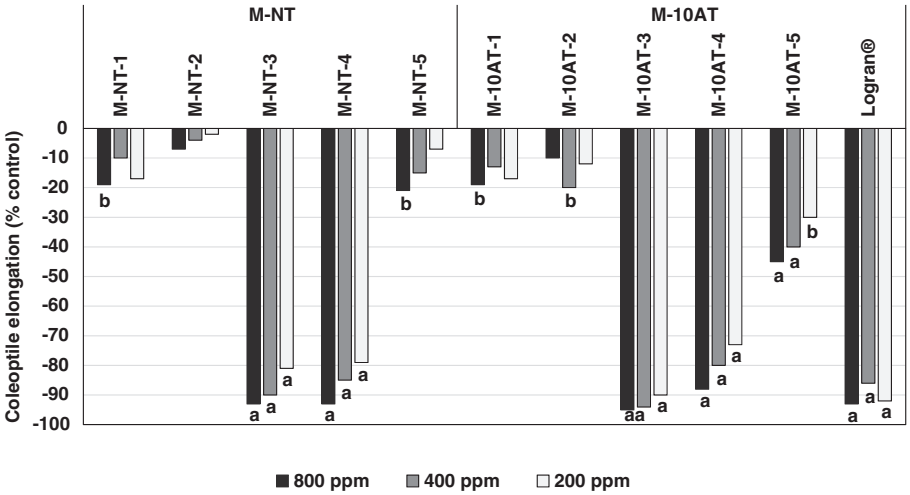

Figure 4. Bioassay with etiolated wheat coleoptiles on the fractions of MeOH–H_2O.

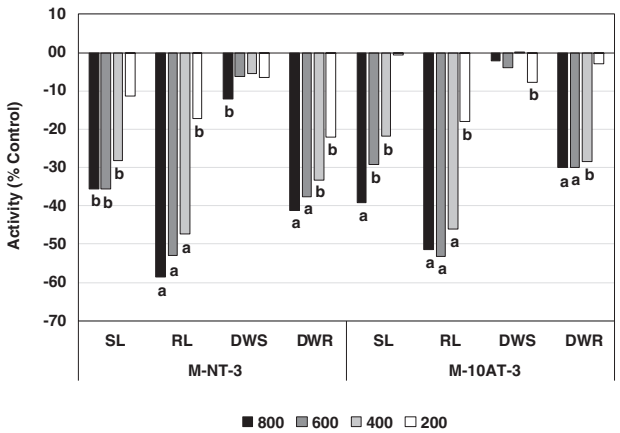

Figure 5. Phytotoxic bioassay on transgenic soybean seedlings on the fractions from MeOH–H_2O. The parameters evaluated were root length (RL), shoot length (SL), dry weight root (DWR) and dry weight shoot (DWS).

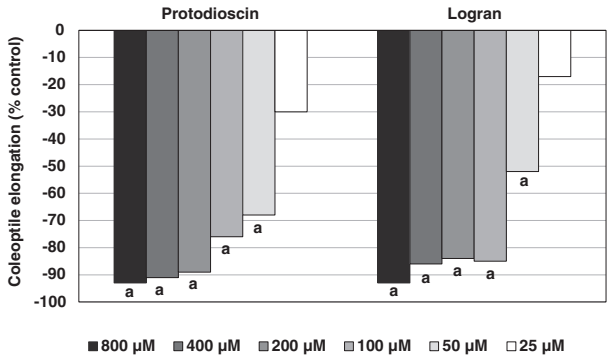

Figure 6. Bioassay with etiolated wheat coleoptiles on protodioscin.

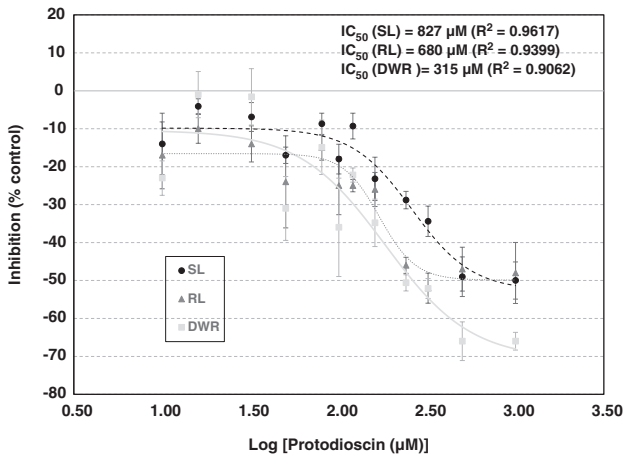

Figure 7. Dose–response curves for protodioscin in phytotoxicity bioassay on transgenic soybean seedlings.

The extracts that showed the highest inhibition levels in the coleoptile bioassay were those obtained with MeOH, which showed the highest inhibition values close to 90% at 800 ppm. The DCM extracts showed lower inhibitory activity. The similarity in the results obtained with extracts M-NT and M-10AT (with and without the application of glyphosate) shows that the presence or absence of herbicide is not a relevant factor in this bioassay and *U. ruziziensis* probably has in its chemical composition substances that are capable of inhibiting the growth of coleoptiles.

In view of the results outlined above, the MeOH extracts were selected to carry out the bioassay-guided isolation. Two materials from *Urochloa* were used in the fractionation, i.e. without the application of glyphosate (M-NT) and 10 days after treatment with glyphosate (M-10AT). Each material was fractionated with increasing H_2O-to-MeOH ratios to give five fractions for each sample. All of these fractions were subjected to the coleoptile bioassay. The results are shown in Fig. 4. It can be seen that the fractions extracted with mixtures of 50% (M-NT-3 and M-10AT-3) and 75% (M-NT-4 and M-10AT-4) MeOH–H_2O were the most active, with values comparable to those of the commercial herbicide Logran® for the two types of material. The inhibition values obtained were above 90% for the highest concentrations tested, with levels above 80% maintained at the lowest concentrations. This similarity in the results supports the hypothesis that the observed activity is related to the chemical composition of the plant material and not to the application of glyphosate.

To test the above hypothesis, the two most abundant active fractions (50% in MeOH), material both without glyphosate (M-NT-3) and with glyphosate (M-10AT-3), were subjected to a soybean

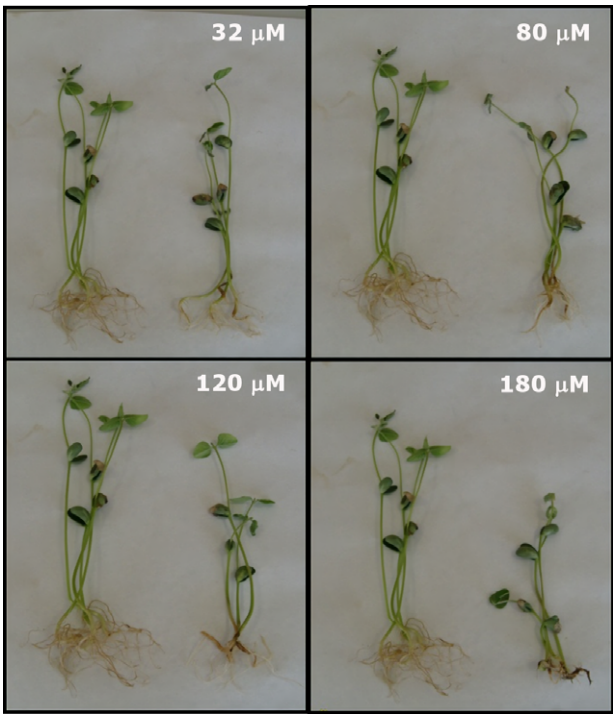

Figure 8. Images of necrosis observed in soybean roots.

seedling bioassay. The results are shown in Fig. 5. It can be seen that there is a significant effect on soybean seedlings in terms of the lengths of both the aerial parts (SL) and the roots (RL), the latter of which is the most sensitive parameter. Inhibition values over 50% at a concentration of 600 ppm were observed for the two types of plant material.

Due to the similarity in the results obtained for both plant materials, the sample treated with glyphosate was chosen to continue the isolation sequence. This selection was made in order to mimic more accurately the problem under investigation, namely the yield loss associated with *U. ruziziensis* waste once desiccated with glyphosate. Therefore, the fractions obtained with 50% MeOH (M-10AT-3, 3.0 g) and 75% MeOH (M-10AT-4, 390 mg) were selected.

Compound **1** was isolated from fraction M-10AT-3 and the spectroscopic data correspond to those described in the literature for protodioscin, a steroidal saponin isolated for the first time from *U. ruziziencis*.[43] Fraction M-10AT-4 afforded, along with **1**, a mixture of **2** and **3**, which were identified as steroid saponins derived from microbial conversion of methylprotodioscin by *Penicillium melinii*.[44]

The major compound **1** has been hypothesized to be responsible for the activity of sunflower crop using desiccated *U. ruziziensis* as cover.[45] In order to verify this hypothesis, this compound was subjected to an activity study. Thus, **1** was tested in both bioassays to assess the activity profile. The results obtained in the coleoptile bioassay are shown in Fig. 6. It can be seen that the inhibitory effect is adjusted to a sigmoidal curve, with an IC_{50} of 27.35 µM ($R^2 = 0.9688$) calculated for protodioscin and 46.92 µM ($R^2 = 0.9966$) for the commercial herbicide. Regarding the soybean seedling bioassay, the means of the effects observed in the two tests were analysed together. Regression analysis on the data for SL, RL and DWR, depending on the concentration of protodioscin (Fig. 7), allowed the inhibitory dose for protodioscin to be calculated as 827 µM ($R^2 = 0.9617$), 680 µM ($R^2 = 0.9399$) and 315 µM ($R^2 = 0.9062$), respectively.

In this assay a very pronounced necrosis was observed in the main root with increasing concentrations of protodioscin (16, 32, 80, 120 and 240 µM; Fig. 8), with strong attenuation and length reduction. In addition, malformations of secondary roots and a reduction in the length of the seedlings were also observed.

4 CONCLUSIONS

Comparison of the results obtained in the bioassay of transgenic soybean seedlings for all products tested (Fig. 9) clearly indicates that protodioscin is the only compound that has a significant phytotoxic effect on transgenic soybean seedlings. As previously mentioned, the activities of phenolic compounds do not explain the observed effects.

Triterpenoid saponins have a wide range of pharmacological properties.[46–48] These kinds of compounds have also been reported to have detrimental effects on plant growth when released into the soil.[49–51] These compounds have recently been investigated for their phytotoxic activity.[52] As a result, a long glycosidic chain linked at C-3 of the aglycone moiety was suggested as a key factor for phytotoxicity. In contrast, the relationship between aglycone structure and phytotoxicity remains unclear.[53]

Protodioscin is a bidesmosidic saponin formed by a hydrophobic moiety of the furostanol type and two sugar fragments. The structural characteristics of these compounds mean that they are readily soluble in water and, therefore, easy leaching to the soil is possible once the *U. ruziziensis* plants have been desiccated with

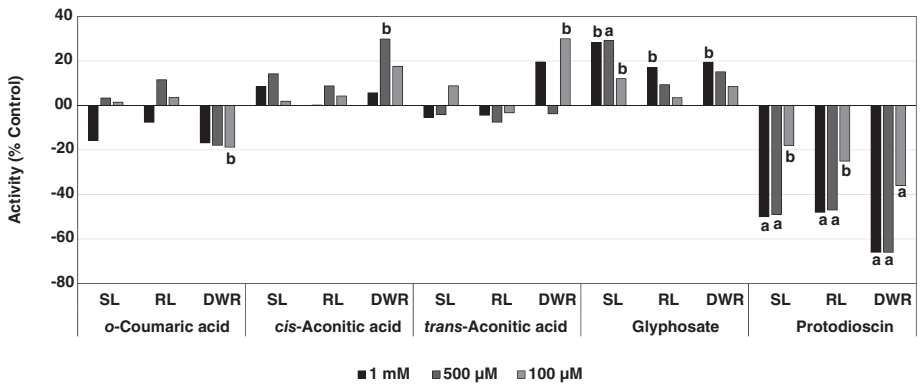

Figure 9. Comparison of results obtained in bioassay on transgenic soybean seedlings. The parameters evaluated were root length (RL), shoot length (SL) and dry weight root (DWR).

glyphosate and exposed to rain.[45] This release into the soil and the phytotoxic effects shown here provide a plausible explanation for the decrease in production yield observed in transgenic soybean crops after desiccation of *U. ruziziensis*.

ACKNOWLEDGEMENT

This research was supported by the Ministerio de Economía y Competitividad (MINECO) (project AGL2013-42238-R).

REFERENCES

1 Peixoto MF and Souza IF, Effects of imazamox and crop residues of sorghum (*Sorghum bicolor* (L.) Moench) on weed control in soybean (*Glycine max* (L.) Merr.) under no-till system. *Cienc Agrotecnol* **26**:252–258 (2002).
2 Barreto RC, Madari BE, Maddock JEL, Machado PLOA, Tortes E, Franchini J *et al.*, The impact of soil management on aggregation, carbon stabilization and carbon loss as CO_2 in the surface layer of a Rhodic Ferralsol in southern Brazil. *Agric Ecosyst Environ* **132**:243–251 (2009).
3 Bayer C, Martin-Neto L, Mielniczuk J, Pavinato A and Dieckow J, Carbon sequestration in two Brazilian Cerrado soils under no-till. *Soil Tillage Res* **86**:237–245 (2006).
4 Novaes P, Molinillo JMG, Varela RM and Macías FA, Ecological phytochemistry of Cerrado (Brazilian savanna) plants. *Phytochem Rev* **12**:839–855 (2013).
5 Nunes UR, Andrade Júnior VC, Silva EdB, Santos NF, Costa HAO and Ferreira CA, Covering crops straw production and common bean productivity in no-tillage system. *Pesqu Agropecu Bras* **41**:943–948 (2006).
6 Cobucci T, Manejo integrado de plantas daninhas em sistema de plantio direto, in *Manejo Integrado Fitossanidade: Cultivo Protegido, Pivo Central e Plantio Direto*, ed. by Zambolim L. UFV, Viçosa, pp. 583–624 (2001).
7 Crusciol CAC, Nascente AS, Borghi E, Soratto RP and Martins PO, Improving soil fertility and crop yield in a tropical region with palisadegrass cover crops. *Agron J* **107**:2271–2280 (2015).
8 Merlin A, Rosolem CA and He ZL, Non-labile phosphorus acquisition by *Brachiaria*. *J Plant Nutr* **39**:1319–1327 (2016).
9 de Carvalho AM, Coelho MC, Dantas RA, Fonseca OP, Júnior RG and Figueiredo CC, Chemical composition of cover plants and its effect on maize yield in no-tillage systems in the Brazilian savanna. *Crop Pasture Sci* **63**:1075–1081 (2013).
10 Maciel CDG, Corrêa MR, Alves E, Negrisoli E, Velini ED, Rodrigues JD *et al.*, Influence of straw management of *Brachiaria decumbens* on the initial development of *Glycine max* and *Euphorbia heterophylla*. *Planta Daninha* **21**:365–373 (2003).
11 Freitas Leal AJ, Rebuá Rodrigues L, Lazarini E, Gomes Junior FG and Muraishi CT, Management of plant species soil cover and productivity of corn and soybeans. *Acta Sci Biol Sci* **27**:199–206 (2005).
12 Nunes AS, Timossi PC, Pavani MCMD and Alves PLCA, *Brachiaria decumbens* management times in soybean under no tillage system. *Planta Daninha* **27**:297–302 (2009).
13 Oliveira Jr RS, Constantin J, Costa JM, Cavalieri SD, Arantes JGZ, Alonso DG *et al.*, Interaction between burndown systems and post-emergence weed control affecting soybean development and yield. *Planta Daninha* **24**:721–732 (2006).
14 Santos JB, Santos EA, Fialho CMT, Silva AA and Freitas MAM, Influence of desiccation time before sowing on the development of soybean resistant to glyphosate. *Planta Daninha* **25**:869–875 (2007).
15 Scognamiglio M, D'Abrosca B, Fiumano V, Chambery A, Severino V, Tsafantakis N *et al.*, Oleanane saponins from *Bellis sylvestris* Cyr. and evaluation of their phytotoxicity on *Aegilops geniculata* Roth. *Phytochemistry* **84**:125–134 (2012).
16 Hancock C, Barlow H and Lacey H, The East Malling coleoptile straight growth test method. *J Exp Bot* **15**:166–176 (1964).
17 Nitsch JP and Nitsch C, Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight-growth test for auxins. *Plant Physiol* **31**:94–111 (1956).
18 Macias FA, Castellano D and Molinillo JMG, Search for a standard phytotoxic bioassay for allelochemicals. Selection of standard target species. *J Agric Food Chem* **48**:2512–2521 (2000).
19 Martín A and Luna JD, *Bioestadística Para las Ciencias de la Salud*. Ediciones Norma, Madrid (1990).
20 *Prism v. 4.0*, GraphPad Software Inc., San Diego, CA (2003).
21 Bohm GMB, Scheneider L, Castilhos D, Agostinetto D and Rombaldi CV, Weed control, biomass and soil microbial metabolism in relation to the application of glyphosate and imazethapyr on soybean crop. *Semina Cienc Agrar* **32**:919–929 (2011).
22 Reddy KN and Zablotowicz RM, Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. *Weed Sci* **51**:496–502 (2003).
23 Moorman TB, Becerril JM, Lydon J and Duke SO, Production of hydroxybenzoic acids by *Bradyrhizobium japonicum* strains after treatment with glyphosate. *J Agric Food Chem* **40**:289–293 (1992).
24 Kaps MA and Kuhns LJ, Glyphosate transfer between plants via mycorrhizal fungi. *HortScience* **22**:1128–1128 (1987).
25 Duke SO, Glyphosate degradation in glyphosate-resistant and -susceptible crops and weeds. *J Agric Food Chem* **59**:5835–5841 (2011).
26 Reddy KN, Rimando AM and Duke SO, Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. *J Agric Food Chem* **52**:5139–5143 (2004).
27 Duke SO, Rimando AM, Pace PF, Reddy KN and Smeda RJ, Isoflavone, glyphosate, and aminomethylphosphonic acid levels in seeds of glyphosate-treated, glyphosate-resistant soybean. *J Agric Food Chem* **51**:340–344 (2003).
28 Velini ED, Alves E, Godoy MC, Meschede DK, Souza RT and Duke SO, Glyphosate applied at low doses can stimulate plant growth. *Pest Manage Sci* **64**:489–496 (2008).
29 Morandi D, Effects of xenobiotics on endomycorrhizal infection and isoflavonoid accumulation in soybean roots. *Plant Physiol Biochem* **27**:697–701 (1989).
30 Barnes JP and Putnam AR, Rye residues contribute weed suppression in no-tillage cropping systems. *J Chem Ecol* **9**:1045–1057 (1983).
31 Souza LS, Velini ED, Martins D and Rosolem CA, Allelopathic effects of *Brachiaria decumbens* on the initial development of seven crops. *Planta Daninha* **24**:657–668 (2006).
32 Voll E, Gazziero DLP and Adegas FS, Aconitic acid on seeds of weed species from different locations. *Planta Daninha* **28**:13–22 (2010).
33 Chou C-H and Patrick ZA, Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. *J Chem Ecol* **2**:369–387 (1976).
34 Kobayashi A and Kato-Noguchi H, Phytotoxic substance with allelopathic activity in *Brachiaria decumbens*. *Nat Prod Commun* **10**:761–763 (2015).
35 Souza Filho APS, Pereira AAG and Bayma JC, Allelochemical produced by the forage grass *Brachiaria humidicola*. *Planta Daninha* **23**:25–32 (2005).
36 Jilani G, Mahmood S, Chaudhry AN, Hassan I and Akram M, Allelochemicals: sources, toxicity and microbial transformation in soil – a review. *Ann Microbiol* **58**:351–357 (2008).
37 Weir TL, Park SW and Vivanco JM, Biochemical and physiological mechanisms mediated by allelochemicals. *Curr Opin Plant Biol* **7**:472–479 (2004).
38 Liebl RA and Worsham AD, Inhibition of pitted morning glory (*Ipomoea lacunosa* L.) and certain other weed species by phytotoxic components of wheat (*Triticum aestivum* L.) straw. *J Chem Ecol* **9**:1027–1043 (1983).
39 Guenzi WD and McCalla TM, Phenolic acids in oats, wheat, sorghum and corn residues and their phytotoxicity. *Agron J* **58**:303–304 (1966).
40 Cochran VL, Elliott LF and Papendick RI, The production of phytotoxins from surface crop residues. *Soil Sci Soc Am J* **41**:903–908 (1977).
41 Voll E, Franchini JC, Da Cruz RT, Gazziero DLP, Brighenti AM and Adegas FS, Chemical interactions of *Brachiaria plantaginea* with *Commelina bengalensis* and *Acanthospermum hispidum* in soybean cropping systems. *J Chem Ecol* **30**:1467–1475 (2004).
42 Cutler SJ, Hoagland RE and Cutler HG, Evaluation of selected pharmaceuticals as potential herbicides: bridging the gap between agrochemicals and pharmaceuticals, in *Allelopathy in Ecological Agriculture and Forestry*, ed. by Narwal SS, Hoagland RE, Dilday RH and Reigosa RMJ. Springer, Berlin, pp. 129–137 (2000).
43 De Combarieu E, Fuzzati N, Lovati M and Mercalli E, Furostanol saponins from *Tribulus terrestris*. *Fitoterapia* **74**:583–591 (2003).

44 He X, Qiao A, Liu B, Wang X, Wang G, Qu G *et al.*, Bioconversion of methyl protodioscin by *Penicillium melinii* cells. *Enzyme Microb Technol* **38**:400–406 (2006).

45 Giancotti PRF, Nepomuceno MP, Alves PLCA and Yamauti MS, Ideal desiccation periods of *Urochloa ruziziensis* for a no-till sunflower crop. *Int J Plant Prod* **9**:39–50 (2015).

46 Shen J, Yang X, Meng Z and Guo C, Protodioscin ameliorates fructose-induced renal injury via inhibition of the mitogen activated protein kinase pathway. *Phytomedicine* **23**:1504–1510 (2016).

47 Wang P, Hao J, Zhang X, Wang C, Guan H and Li M, Synthesis of furostanol glycosides: discovery of a potent α-glucosidase inhibitor. *Org Biomol Chem* **14**:9362–9374 (2016).

48 Zhang X, Xue X, Xian L, Guo Z, Ito Y and Sun W, Potential neuroprotection of protodioscin against cerebral ischemia-reperfusion injury in rats through intervening inflammation and apoptosis. *Steroids* **113**:52–63 (2016).

49 Marchaim U, Werker E and Thomas WDE, Changes in the anatomy of cotton seed coats caused by Lucerne saponins. *Bot Gaz* **135**:139–146 (1974).

50 Oleszek W, Allelopathic potentials of alfalfa (*Medicago sativa*) saponins: their relation to antifungal and hemolytic activities. *J Chem Ecol* **19**:1063–1074 (1993).

51 Oleszek W, Jurzysta M and Górski PM. Alfalfa saponins: the allelopathic agents, in *Allelopathy: Basic and Applied Aspects*, ed. by Rizvi SJH and Rizvi V. Springer, Dordrecht, pp. 151–167 (1992).

52 Hernández-Carlos B, González-Coloma A, Orozco-Valencia ÁU, Ramírez-Mares MV, Andrés-Yeves MF and Joseph-Nathan P, Bioactive saponins from *Microsechium helleri* and *Sicyos bulbosus*. *Phytochemistry* **72**:743–751 (2011).

53 Perez AJ, Simonet AM, Calle JM, Pecio L, Guerra JO, Stochmal A *et al.*, Phytotoxic steroidal saponins from *Agave offoyana* leaves. *Phytochemistry* **105**:92–100 (2014).