J Plant Growth Regul (2015) 34:339–349
DOI 10.1007/s00344-014-9469-2

Phytotoxicity and Identification of Secondary Metabolites of *Sapindus saponaria* L. Leaf Extract

Patrícia Umeda Grisi · Moacir Rossi Forim · Eveline Soares Costa · Simoni Anese · Marcos Fernando Franco · Marcos Nogueira Eberlin · Sônia Cristina Juliano Gualtieri

Received: 31 August 2014 / Accepted: 7 November 2014 / Published online: 18 December 2014
© Springer Science+Business Media New York 2014

Abstract The aim of this study was to evaluate and quantify the phytotoxic effects of hexane and ethyl acetate fractions obtained from a leaf extract of *Sapindus saponaria* on the germination and seedling growth of *Euphorbia heterophylla* (wild poinsettia) and *Echinochloa crus-galli* (barnyardgrass) and to isolate and identify the major bioactive compounds. A crude ethanol extract was prepared from 100 g of dry plant material in 500 mL of ethanol. The extract was fractionated by liquid–liquid extraction, and the hexane and ethyl acetate fractions were solubilized at concentrations of 0.625, 1.25, 2.5, and 5.0 mg mL^{-1}. The effect of these fractions was compared with the oxyfluorfen herbicide in bioassays. The hexane and ethyl acetate fractions inhibited germination, induced abnormalities, and reduced seedling growth of *E. crus-galli* and *E. heterophylla* with concentration-dependent effects. The phytotoxicity of the fractions ranged according to the receptor species, and the ethyl acetate fraction showed a greater inhibitory effect than the hexane fraction on seedling development. For both species, the oxyfluorfen herbicide inhibited mainly shoot growth, whereas the plant extracts inhibited seedling root growth. The *S. saponaria* fractions caused a reduction of more than 50 % in the size of metaxylem cells of *E. heterophylla* roots. The ethyl acetate fraction obtained from *S. saponaria* leaves was subjected to fractionation, and the substance isolated was identified as 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid. This compound also inhibited the germination and seedling growth of *E. crus-galli* and *E. heterophylla*, but the ethyl acetate fraction was more potent in reducing seedling growth.

Keywords Allelochemicals · *Echinochloa crus-galli* · *Euphorbia heterophylla* · Sapindaceae · Terpene

Electronic supplementary material The online version of this article (doi:10.1007/s00344-014-9469-2) contains supplementary material, which is available to authorized users.

P. U. Grisi (✉) · S. C. J. Gualtieri
Department of Botany, Federal University of São Carlos, Rodovia Washington Luiz, Km 235, Caixa Postal 676, São Carlos, SP 13565-905, Brazil
e-mail: patriciaumeda@hotmail.com

M. R. Forim · E. S. Costa
Department of Chemistry, Federal University of São Carlos, Rodovia Washington Luiz, Km 235, São Carlos, SP 13565-905, Brazil
e-mail: mrforim@ufscar.br

S. Anese
Science and Technology of Mato Grosso - Campus, Federal Institute of Education, MT 235 Km 12, Campo Novo do Parecis, MT 78360-000, Brazil
e-mail: simonianese@yahoo.com.br

M. F. Franco · M. N. Eberlin
ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas - UNICAMP, Campinas, SP 13083-970, Brazil
e-mail: marcosfranco05@gmail.com

Introduction

Bioactive phytochemicals released into the environment can influence plant growth and development (Sodaeizadeh and others 2009). There is an increasing evidence that these plant chemicals can suppress the germination and growth of different weed species (Tawaha and Turk 2003; Sampietro and Vattuone 2006). *Echinochloa crus-galli* (L.) P. Beauv. (Poaceae, barnyardgrass) and *Euphorbia heterophylla* L. (Euphorbiaceae, wild poinsettia) are weeds with a very common occurrence in Brazil and cause large impacts

on agricultural systems (Aliotta and others 2006; Vidal and others 2007). The main control method for these species is the application of synthetic herbicides, with consequent environmental impacts and selection of biotypes with herbicide resistance (Batish and others 2007; Grisi and others 2013). Indeed, chemical control of weeds is the method most widely used due to its high efficiency and practicality (Panozzo and others 2014). However, the intense application of pesticides has resulted in environmental damage, acting on the balance of microorganisms and causing changes in the physico-chemical properties of the soil, resulting in decreased crop productivity (Chou 1999).

The incorporation of substances with allelopathic activity in agriculture can reduce environmental pollution and increase agricultural production for sustainable weed management (Parvez and others 2003; He and others 2004; Sodaeizadeh and others 2009). Plants are able to develop defense mechanisms based on the synthesis of certain secondary metabolites, which when released into the environment will interfere at some stage of the life cycle of other plants (Marco and others 2012). The accumulation of substances with allelopathic effects has been found in all plant organs, with a tendency for accumulation in the leaves (Dorning and Cipollini 2006; Khan and others 2011). However, allelochemicals vary in composition, concentration, and plant location (Hong and others 2004). Their phytotoxic effects are mediated by chemical substances belonging to different classes of compounds, such as phenols, terpenes, alkaloids, polyacetylenes, fatty acids, peptides, glycosides, and saponins (Wink 2003).

Species from the Sapindaceae family are known for their traditional medicinal uses as a diuretic, stimulant, expectorant, natural surfactant, sedative, anthelmintic, and agent against stomachache and dermatitis in many parts of the world (Tsuzuki and others 2007). Chemical investigations of this family have led to the isolation of saponins, diterpenes, and flavonoids, among other secondary metabolites (Pelegrini and others 2008). *Sapindus saponaria* L. (soapberry, Sapindaceae) is a medium size tree distributed all over the tropics and usually occurs in rain and semi-deciduous forests (Murgu and Rodrigues-Filho 2006; Tsuzuki and others 2007). The main bioactive substances found in its fruits are saponins and acyclic sesquiterpene oligoglycosides (Murgu and Rodrigues-Filho 2006). Despite the potential importance of *Sapindus* plants as a good source of bioactive saponins, there is a lack of detailed studies on the chemical composition of *S. saponaria* (Murgu and Rodrigues-Filho 2006). In our previous report, we found that aqueous extracts of *S. saponaria* leaves exhibited inhibitory effects on the germination and seedling growth of *E. crus-galli* and *Ipomoea grandifolia* (Dammer) O'Donell (Grisi and others 2012). Based on this information, *S. saponaria* leaves may produce biologically active compound(s) and possess potential sources of natural herbicides.

Thus, the aims of this study were (1) to evaluate and quantify the phytotoxic effects of hexane and ethyl acetate fractions from an ethanolic extract of *S. saponaria* leaves on diaspore germination and seedling growth of *E. heterophylla* and *E. crus-galli*; (2) to isolate and identify bioactive compounds; and (3) to examine the phytotoxicity of the isolated compound compared with its derivative mixture.

Materials and Methods

Fraction Preparation

Mature leaves (dark green color and papyraceous texture) of *S. saponaria* were collected in São Carlos-SP, (22°02′S and 47°52′W), Brazil, in May 2011. The region is characterized by Aw climate (Köppen 1948), with dry winters (April to September) and wet summers (October to March). The collected leaves were dried at 40 °C for 72 h and ground using an industrial mill.

The ethanolic extract was prepared in the proportion of 100 g of dry plant material in 500 mL of ethanol. The plant material was exhaustively extracted with ethanol under dark and cold conditions. After 72 h, the solution was filtered and concentrated in a rotary evaporator under reduced pressure, resulting in a crude ethanol extract. The resulting crude extract (60 g) was suspended in methanol (MeOH) and deionized water at a ratio of 1:3 (w/w). This solution was fractionated by liquid–liquid extraction using hexane, ethyl acetate (EtOAc), and butanol solvents. The solutions obtained were evaporated under reduced pressure producing hexanic (22.27 g), EtOAc (5.43 g), butanolic (10.92 g), and aqueous (11.34 g) fractions. Each fraction was subjected to a wheat coleoptile bioassay. The hexane and EtOAc fractions, which showed higher inhibitory activity, were solubilized in a buffer solution (10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH, pH 6) and DMSO (dimethyl sulfoxide, 5 μL mL^{-1}) at concentrations 5.0, 2.5, 1.25, and 0.625 mg mL^{-1} (Macías and others 2010). Germination and seedling growth bioassays included two controls: a negative control with the buffer solution and DMSO and a positive control with the oxyfluorfen herbicide (240 g.i.a. L^{-1}) at doses of 1.0 and 2.0 L ha^{-1}. Oxyfluorfen is a selective herbicide, and not systemic, and is used for the weed control (monocotyledonous and eudicotyledonous) in pre-emergence or early post-emergence applications (Rodrigues and Almeida 2011).

Germination and Seedling Growth Bioassay

The hexane and EtOAc fractions from the ethanol extract of *S. saponaria* leaves were applied on *E. heterophylla* and *E. crus-galli* diaspores. The bioassays were conducted in Petri dishes (9 cm of diameter) with two sheets of filter paper moistened with 5 mL of the fractions, herbicide, or buffer solution and DMSO (5 μL mL^{-1}). The experimental design was completely randomized, using four replicates of 30 diaspores. The experiment was conducted in a germination chamber at 25 °C, with a photoperiod of 12 h. The germination criterion was embryo protrusion, which was evaluated every 12 h during the first 7 days of the experiment and at intervals of 24 h thereafter until the stabilization of germination. Germinability, mean germination time and rate (Maguire's index) were calculated according to Ranal and Santana (2006).

For the analysis of *E. heterophylla* and *E. crus-galli* seedling growth, diaspores were previously germinated in distilled water. Only seedlings with 3 mm-long roots were selected and transferred to transparent plastic boxes (13 × 8 × 3 cm) containing, as a substrate, filter paper moistened with 5 mL of the fractions, herbicide, or buffer solution and DMSO (5 μL mL^{-1}) under the same concentrations adopted for the germination test. The boxes were kept in a germination chamber at 25 °C, with a photoperiod of 12 h. The experimental design was completely randomized, with four replicates of 10 seedlings. After 7 days, the seedlings were classified as normal or abnormal (Brasil 2009), and the shoot and primary root lengths of the seedlings were measured using a caliper.

Examination of Metaxylem Cells

Euphorbia heterophylla seedlings were grown in negative control solutions and in the hexane and EtOAc fractions of *S. saponaria* leaves under the same conditions adopted for the growth bioassay. After 4 days, the primary root segments of the seedlings were removed and immersed in 70 % alcohol (Gatti and others 2010). The modified Fuchs staining method was used (Kraus and Arduin 1997), where the roots were immersed in alcohol (70 %) for 5 days and placed in a solution of 25 % NaOH at 60 °C for 48 h, until the material was clarified.

Then, the root segments were immersed in safranin ($C_{20}H_{19}N_4C_1$) and caustic soda (10 % NaOH) for 24 h at 60 °C. After staining, the material was mounted on glass slides in Apathy's syrup (Kraus and Arduin 1997), with the roots, for observation under an optical microscope (Olympus-BX41) coupled to a camera (Sony CCD-IRIS). Four primary roots of *E. heterophylla* seedlings grown in different concentrations of the fractions and control solutions were used. Half of the length of each root from the central region upward was photographed. From each photograph, 15 central cells of the metaxylem were measured at 20× magnification (Image Pro Plus 5.0 program) (Gatti and others 2010).

Fractionation and Identification of Secondary Metabolites

The EtOAc fraction (3.0 g), which was selected from the results obtained by the bioassays, was suspended in MeOH and deionized water at a ratio of 1:3 (w/w). This solution was fractionated by liquid–liquid extraction using hexane, dichloromethane (CH_2Cl_2), and EtOAc. The solutions were evaporated under reduced pressure producing hexane (89.7 mg), CH_2Cl_2 (577.2 mg), EtOAc (624.4 mg), and aqueous (964.8 mg) fractions. According to observations by silica gel thin-layer chromatography (TLC), the CH_2Cl_2 and EtOAc fractions were pooled and subjected to silica gel (230–400 mesh) column chromatography and eluted with hexane/acetone mixtures of increasing polarity followed by methanol. This procedure yielded 41 sub-fractions, which were monitored by TLC. According to TLC observations, the sub-fractions F19 (hexane/acetone 7:3, 3.2 mg), F20 (hexane/acetone 6:4, 3.9 mg), F21 (hexane/acetone 6:4, 54.3 mg), F22 (hexane/acetone 6:4, 5.1 mg), F23 (hexane/acetone 1:1, 3.0 mg), and F24 (hexane/acetone 1:1, 3.4 mg) were pooled and subjected to analyses by 1D and 2D Nuclear Magnetic Resonance (NMR) (Bruker 600 MHz, 14.1 T) and Mass Spectrometry (LC–MS and LC–MS/MS) for identification of the purified substance.

To analyze the compound through LC–MS and LC–MS/MS, the sample was solubilized in water:acetonitrile (1:1; v/v) to a concentration of 650 μg mL^{-1}. Afterward, this solution was diluted to 32.5 μg mL^{-1}. Then, the sample was analyzed using an Agilent 1290 Series Liquid Chromatography apparatus (Agilent Technologies, USA). The analysis was carried out in the reversed-phase gradient mode using a stainless steel Zorbax Extend-C_{18} (1.8 μm, 2.1x50 mm, Agilent Technologies, USA) and a mobile phase consisted of acetonitrile (phase B) and TFA 0.1 % (v/v). The gradient method was as follows: 0–1 min 50 % B; 1–2 min 90 % B; 2–5 min 90 % B. The flow rate was 0.5 mL min^{-1} at 25 °C, and injected volume was 2 μL. Acquisition was carried out using an Agilent ifunnel Q-TOF 6550 LC–MS with source Dual Agilent Jet Stream ESI (Dual AJS-ESI) in the following conditions: drying gas at 280 °C, drying gas flow 14 L min^{-1}, nebulizer at 20 psi; sheath gas at 350 °C; sheath gas flow 12 L min^{-1}, VCap 2500; nozzle voltage 0 V; fragmentor 110 V, OCT 1 RF Vpp 750 V, and collision energy 20 V using N_2. Mass spectrometer parameters acquisition ranged from 50 to 220 *m/z* to MS and targeted MS/MS.

The isolated compound was also subjected to germination and seedling growth bioassays with *E. heterophylla* and *E. crus-galli* under the same conditions described above. The compound was solubilized in a buffer solution and DMSO (5 μL mL^{-1}) at concentrations 1 mM, 300, 100, 30, and 10 μM. This germination bioassay was conducted in Petri dishes (5 cm of diameter) with one sheet of filter paper moistened with 1.5 mL of the compound or control solution. The experimental design was completely randomized, using four replicates of 20 diaspores.

Statistical Analysis

The data were subjected to normality (Shapiro–Wilk) and homogeneity (Levene) tests. When these two assumptions were met, an analysis of variance (ANOVA) was applied, followed by Tukey's test at a significance level of 0.05. Linear or quadratic regression models were adjusted when the ANOVA *F* was significant. The goodness of the models was evaluated by the coefficient of determination (R^2). The data were subjected to a conjoint analysis because the ratio between the larger and smaller residual mean square was not greater than 7 (Pimentel-Gomes 1990).

Results

The hexane fraction of the ethanol extract of *S. saponaria* leaves significantly inhibited the germination process and seedling growth of *E. heterophylla* and *E. crus-galli*. For *E. crus-galli*, the hexane fraction linearly increased the mean germination time (1.77 h for each addition of 1 mg mL^{-1} of fraction); in contrast, germination (60.60 %) and the Maguire's index (0.2602 caryopses h^{-1}) reached minimum values at estimated concentrations of 3.46 and 3.96 mg mL^{-1}, respectively (Fig. 1). The *E. heterophylla* seeds showed minimum values of germinability (45.63 %) and Maguire's index (0.2911 seeds h^{-1}) at estimated concentrations of 4.13 and 3.91 mg mL^{-1}, respectively. Moreover, the mean germination time reached a maximum (55.55 h) at a concentration 3.95 mg mL^{-1} for the hexane fraction (Fig. 1). *E. crus-galli* seedlings grown in solutions containing the hexane fraction had the lowest shoot (24.73 mm) and root (10.31 mm) lengths at concentrations of 3.62 and 3.85 mg mL^{-1}, respectively. However, the shoot (34.12 mm) and root (15.09 mm) lengths of the *E. heterophylla* seedlings were minimal at estimated concentrations of 3.68 and 3.87 mg mL^{-1}, respectively (Fig. 1).

The EtOAc fraction from *S. saponaria* leaves also showed phytotoxic effects on the development of the studied weeds. For *E. crus-galli*, minimum values were observed for germinability (60.90 %) and Maguire's index (0.2465 caryopses h^{-1}) at estimated concentrations of 3.42 and 3.88 mg mL^{-1}, respectively, whereas the mean germination time increased linearly by 2.90 h for each addition of 1 mg mL^{-1} of the fraction (Fig. 2). For *E. heterophylla* seeds seeded with the EtOAc fraction, there was a linear decrease and increase in the germinability and mean germination time (1.59 % and 3.27 h for each addition of 1 mg mL^{-1} of fraction), respectively. At the concentration 4.24 mg mL^{-1}, the EtOAc fraction showed the lowest Maguire's index (0.2856 seed h^{-1}) with the *E. heterophylla* seeds (Fig. 2), indicating that the allelochemicals also had the potential to reduce seed vigor. The lowest shoot lengths of the *E. crus-galli* (36.31 mm) and *E. heterophylla* (33.64 mm) seedlings grown in a solution containing the EtOAc fraction were recorded at concentrations of 4.32 and 3.75 mg mL^{-1}, respectively (Fig. 2). The root lengths of *E. crus-galli* and *E. heterophylla* seedlings showed null values at estimated EtOAc fraction concentrations of 3.31 and 3.53 mg mL^{-1}, respectively (Fig. 2). For both species, a greater inhibitory effect was found on the root system.

The maximum percentage of abnormal seedlings of *E. crus-galli* was recorded at estimated concentrations of 3.95 (55.78 %) and 4.25 mg mL^{-1} (69.21 %) of the hexane and EtOAc fractions, respectively (Figs. 1, 2). The *E. heterophylla* seedlings were more sensitive, with a higher incidence of abnormal seedlings observed at concentrations of 3.56 (73.80 %) and 4.24 mg mL^{-1} (92.96 %) of the hexane and EtOAc fractions, respectively (Figs. 1, 2). For both weeds, the EtOAc fraction resulted in a higher percentage of abnormal seedlings than the hexane fraction (Table 1). The herbicide treatment was more efficient at controlling these weeds, with a higher incidence of abnormal seedlings. However, the effect of the EtOAc fraction was similar to the herbicide on the *E. heterophylla* seedlings (Table 1). The main anomalies observed for the abnormal *E. crus-galli* and *E. heterophylla* seedlings are provided in Table 1.

Because *E. heterophylla* was the most sensitive target species, the anatomical study of its roots allowed better visualization of the action mode of different fractions at the cellular level. The average size of the metaxylem cells of the seedlings grown in control was 400.25 μm (±9.76). However, the *S. saponaria* fractions caused a reduction of more than 50 % in the size of root metaxylem cells (Hexane: 278.27–123.25 μm, EtOAc: 182.51–80.88 μm) (Figs. 3, 4). The metaxylem cell size of *E. heterophylla* roots grown under the influence of the hexane and EtOAc fractions was inversely proportional to fraction concentrations (Fig. 3). For all concentrations tested, the EtOAc fraction showed greater inhibitory effect on metaxylem cell elongation than the hexane fraction, with a minimum size of 80.88 μm at a concentration of 5.0 mg mL^{-1} (Figs. 3, 4).

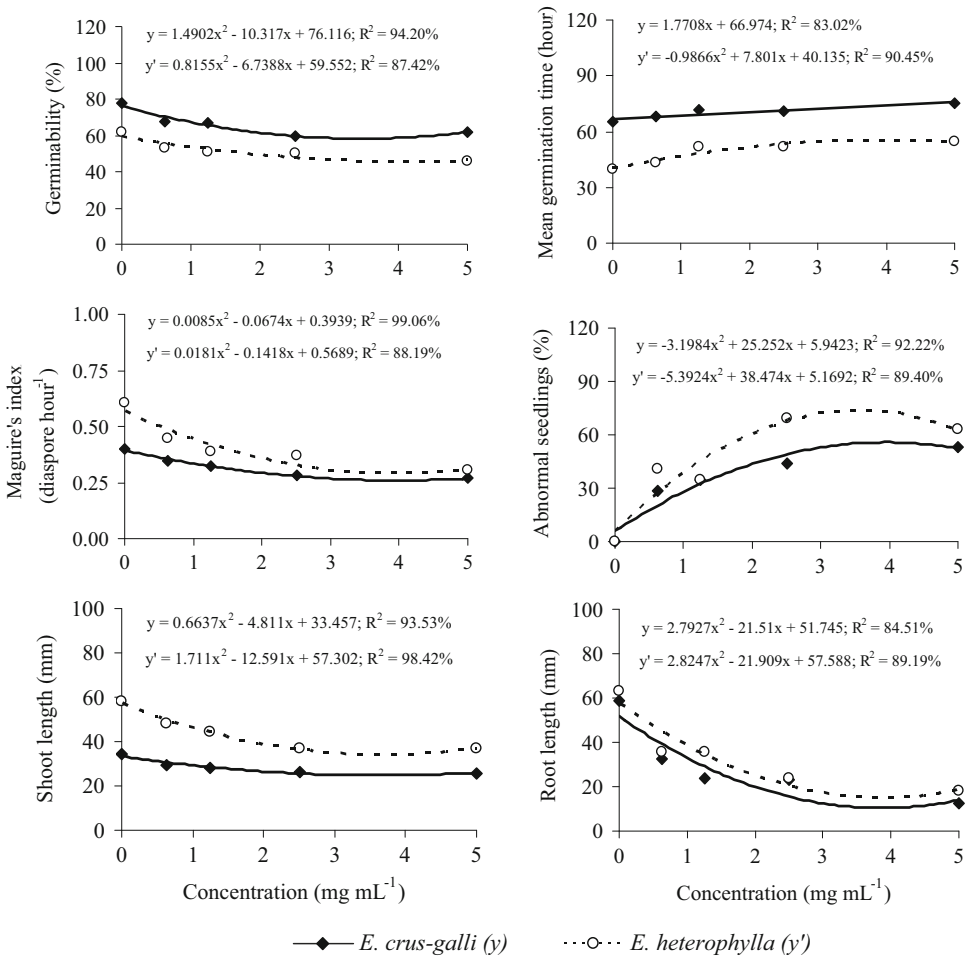

Fig. 1 Germinability, mean germination time, Maguire's index, abnormal seedlings, shoot and root lengths of *E. crus-galli* and *E. heterophylla* seedlings treated with the hexane fraction of *S. saponaria* leaves at different concentrations

Comparing the effect of different fractions obtained from *S. saponaria* leaves with the oxyfluorfen herbicide, it was observed that inhibition (over control) of germinability of *E. crus-galli* caryopses was similar for all treatments (Fig. 6a). For *E. heterophylla*, the herbicide (28.95 %), followed by hexane fraction (18.92 %), showed a greater inhibitory effect on seed germination (Fig. 6b). For both weeds, shoot elongation was more affected by the herbicide, followed by the EtOAc fraction. The inhibitory effect of the hexane fraction and herbicide was similar on root length, whereas the EtOAc fraction showed greater inhibition on this organ (Fig. 6a, b). Thus, the results showed that the EtOAc fraction had a greater inhibitory effect than the hexane fraction on the growth and development of *E. crus-galli* and *E. heterophylla* seedlings.

Based on these results, the EtOAc fraction obtained from *S. saponaria* leaves was subjected to fractionation for the purification and identification of compounds. Using analytical methods such as classical chromatography, subfractions 19–24 showed significant purity for the structural characterization by 1D and 2D NMR. The spectroscopic data indicated the presence of a secondary metabolite named 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid, belonging to the terpenes class (Fig. 5).

The structure of the metabolite isolated indicates a monoterpene acid with spectroscopic characteristics not known in the literature. The ^{1}H NMR (600 MHz, $CDCl_3$) spectrum of the compound showed three singlets at δ1.472 (C-6′, 3H), 1.274 (C-7′, 3H), and 1.786 (C-4, 3H) for methyl groups, a broad singlet at δ 5.690 (C-2, 1H) that corresponds to ethylene hydrogen alpha to an alpha–beta unsaturated carbonyl, two double doublets at δ 1.532 (C-3′; 14.0, 4.0 Hz,1H), 1.780 (C-5′; 14.0, 4.0 Hz,1H) and two double triplets at *δ* 1.980 (C-3′; 14.0, 2.4 Hz,1H), 2.460 (C-5′; 14.0, 2.4 Hz,1H) which corresponding to two methylenic carbons of the cyclopentane, and a multiplet regarding to carbinoic hydrogen at δ4.331 (C-4′; m,1H). The ^{13}C NMR (150 MHz, $CDCl_3$) spectrum of the compound indicated signals at δ182.6 (C-1), 112.0 (C- 2), 172.0 (C-3), 26.9 (C-4), 35.9 (C-1′), 86.7 (C-2′), 47.3 (C-3′), 66.8 (C-4′),45.6 (C-5′), 26.4 (C-6′), and 30.6 (C-7′). Analysis of the 1D and 2D NMR spectra are summarized in the Table 1S

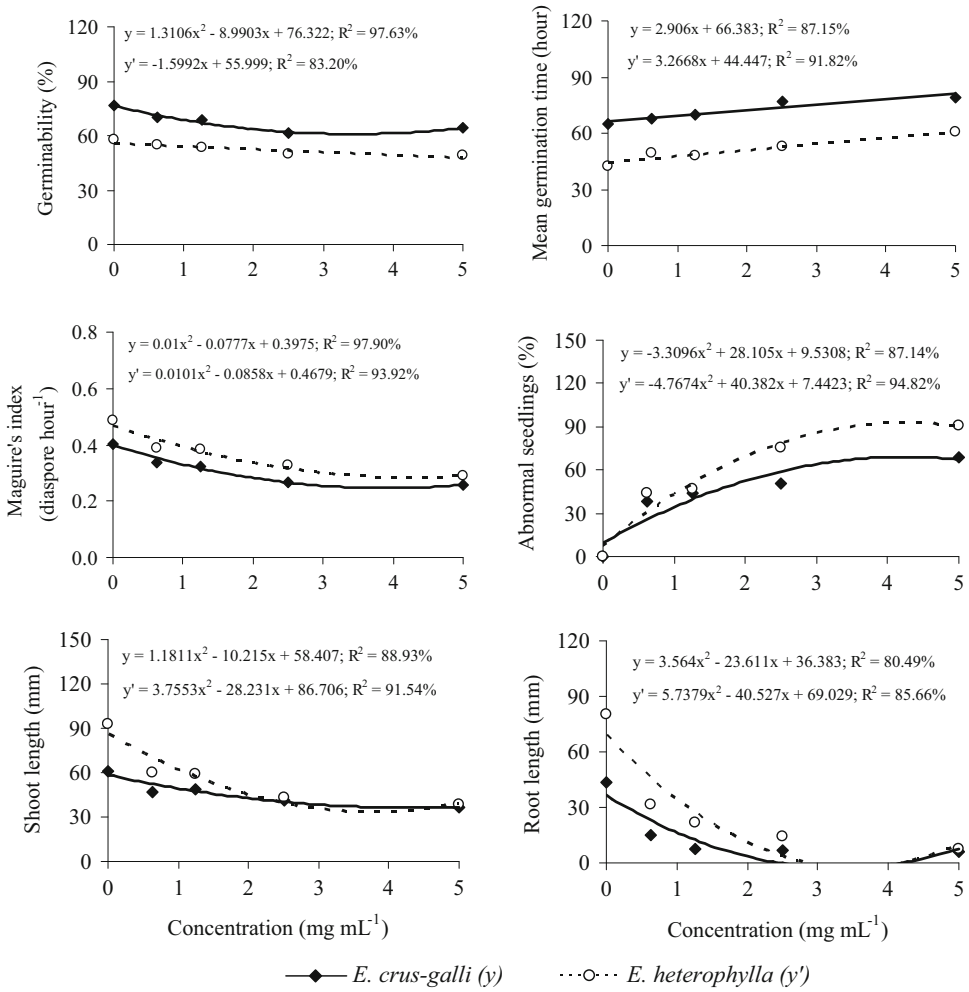

Fig. 2 Germinability, mean germination time, Maguire's index, abnormal seedlings, shoot and root lengths of *E. crus-galli* and *E. heterophylla* seedlings treated with the ethyl acetate fraction of *S. saponaria* leaves at different concentrations

Table 1 Abnormal seedlings of *E. crus-galli* and *E. heterophylla* under the action of the herbicide and the hexane and ethyl acetate fractions of *S. saponaria* leaves and the major anomalies observed

Treatments	Abnormal seedlings (%)	Anomalies
E. crus-galli		
Hexane	40.06 b	Root (stunted, short and necrosed)
Ethyl acetate	50.31 ab	Shoot (short, twisted, deteriorated and absent)
Herbicide	58.75 a	
E. heterophylla		
Hexane	56.53 b	Root (stunted, short, necrosed, with negative geotropism, absence of lateral roots and early development of secondary roots)
Ethyl acetate	69.53 a	
Herbicide	71.87 a	Shoot (short, curved, twisted, deteriorated and gravitropic inversion)

Means followed by the same letter in each column are not significantly different based on Tukey's test, at 0.05 probability

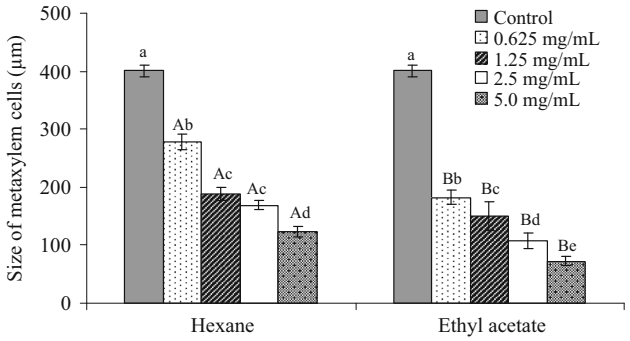

Fig. 3 Size (µm) of the root metaxylem cells of *E. heterophylla* seedlings grown in different concentrations of the hexane and ethyl acetate fractions of *S. saponaria* leaves. Means followed by the *same capital letters* for fractions and *small letters* for concentrations do not differ significantly by Tukey's test at 0.05 % probability. *Vertical bars* represent standard deviations

and Figs. 1S–5S. Considering the number of carbon, hydrogens, and oxygens proposed for the molecule, it was possible to obtain the molecular formula $C_{11}H_{16}O_3$ with a

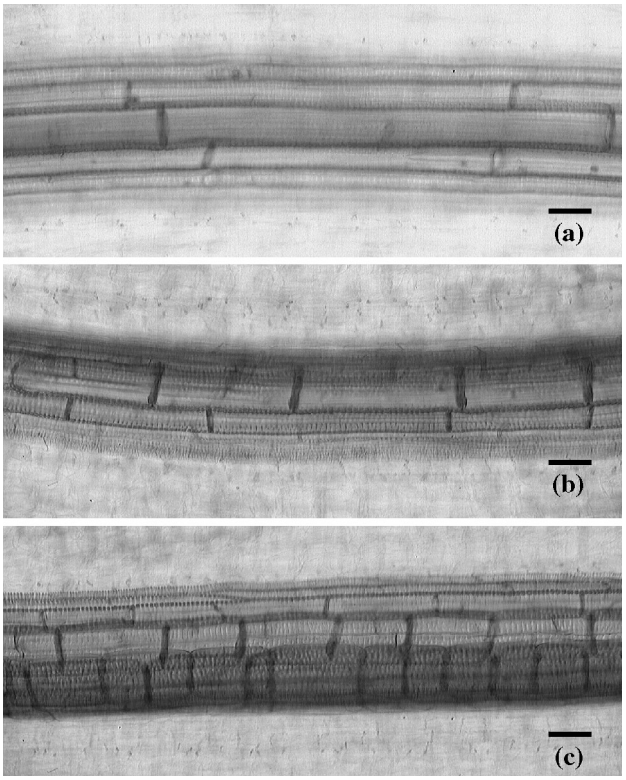

Fig. 4 Photomicrographs of root metaxylem cells of *E. heterophylla* seedlings treated with the control solution (**a**) and the hexane (**b**) and ethyl acetate (c) fractions of *S. saponaria* leaves at a concentration of 5 mg mL^{-1}. *Scale* = 50 μm

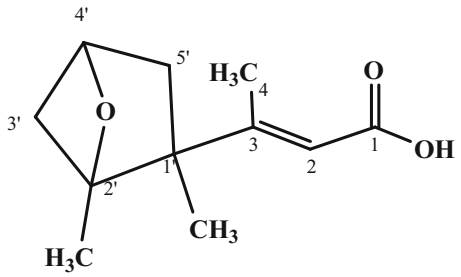

Fig. 5 Chemical structure of the isolated compound 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid

molecular mass of 196 g mol^{-1} and an unsaturation index of 4. The ion *m/z* 197.1165, corresponding to ion $[M + H]^+$, and identified by analysis of *full scan,* was confirmed by high-resolution mass spectrometry (Fig. 6S). The proposed structure was confirmed by analysis of sequential mass spectrometry (MS/MS, Fig. 7S). The analysis of the product ion *m/z* 197.1165 confirmed the ions *m/z* 179.1057, 161.0950, 133.1002, and 107.0850 as being derived from respective product ion (Fig. 7S).

The metabolite 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid inhibited the germination and seedling growth of *E. crus-galli* and *E. heterophylla*, with a concentration-dependent effect, inhibiting mainly root growth (Table 2). This compound (32 %) exerted a stronger inhibitory effect than the herbicide (16 %) and the hexane (17 %) and EtOAc (14 %) fractions on the germination of *E. crus-galli* caryopses. *E. heterophylla* germination was also affected by this compound (15 %), showing greater inhibition than the EtOAc fraction (9.8 %). The isolated compound (54 %) inhibited the root growth of *E. crus-galli*, with an effect similar to the herbicide (59 %) and hexane fraction (61 %). However, *E. heterophylla* seedlings were less affected by the compound (Fig. 6).

Table 2 Effect of 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid isolated from the ethyl acetate fraction of *S. saponaria* leaves on the germination and seedling growth of *E. crus-galli* and *E. heterophylla*

Concentration (M)	Germination (%)	Length (mm)	
		Shoot	Root
E. crus-galli			
Control	68.75 ± 2.50 a	29.25 ± 2.48 a	43.94 ± 5.09 a
10^{-5}	51.25 ± 5.50 ab	24.50 ± 6.06 ab	27.75 ± 8.83 b
3×10^{-5}	47.50 ± 5.50 ab	22.03 ± 1.11 ab	21.68 ± 3.58 bc
10^{-4}	43.75 ± 6.57 b	22.75 ± 0.37 ab	19.27 ± 1.11 bc
3×10^{-4}	45.00 ± 6.12 b	19.50 ± 5.82 b	18.04 ± 4.44 bc
10^{-3}	45.00 ± 3.53 b	16.98 ± 1.78 b	14.55 ± 1.19 c
E. heterophylla			
Controle	65.00 ± 4.08 a	51.33 ± 2.49 a	62.56 ± 2.21 a
10^{-5}	60.00 ± 4.08 ab	44.32 ± 2.28 ab	50.97 ± 1.78 b
3×10^{-5}	56.25 ± 4.73 ab	43.35 ± 10.21 ab	50.01 ± 2.44 b
10^{-4}	55.00 ± 4.08 bc	42.73 ± 1.78 ab	44.59 ± 2.89 c
3×10^{-4}	57.50 ± 3.23 ab	42.40 ± 4.73 ab	44.51 ± 0.92 c
10^{-3}	46.25 ± 2.39 c	38.80 ± 2.14 c	43.97 ± 1.13 c

Means followed by the same letter in each column are not significantly different based on Tukey's test, at 0.05 probability

Discussion

The hexane and EtOAc fractions obtained from the ethanolic extract of *S. saponaria* leaves were phytotoxic and inhibited germination, reduced growth and induced the appearance of abnormalities in *E. crus-galli* and *E. heterophylla* seedlings. For all parameters, the phytotoxicity varied with the species tested, and the inhibitory effect was concentration dependent (Figs. 1, 2). Several studies show that the negative effect of chemical substances on weeds is species-dependent (Prati and Bossdorf 2004; Xuan and others 2005; Sodaeizadeh and others 2009). According to Kobayashi (2004), the susceptibility of a target species to phytotoxic substances under laboratory conditions depends on the physiological and biochemical characteristics of each species.

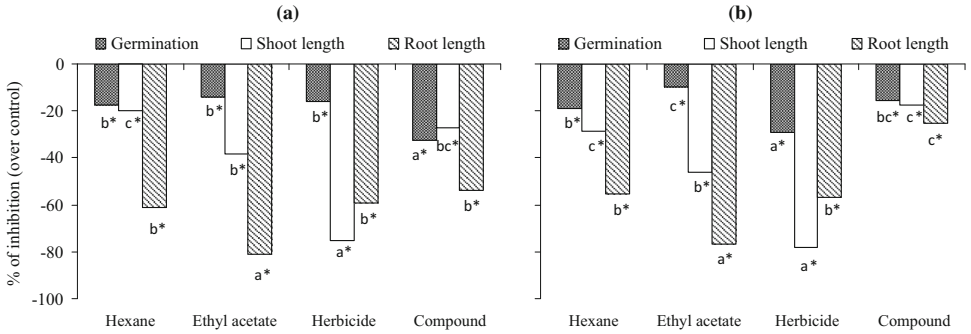

Fig. 6 Inhibitory effects of the hexane and ethyl acetate fractions, herbicide and 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid (isolated compound) from *S. saponaria* leaves on the germination and seedling growth of *E. crus-galli* (**a**) and *E. heterophylla* (**b**). Means followed by the *same letter* in the fractions are not significantly different based on Tukey's test at 0.05 probability. * Significant difference in relation to the control

The effect of *S. saponaria* organic fractions on the germination process showed that allelochemicals may act not only in germinability but also on the mean germination time and vigor of *E. crus-galli* and *E. heterophylla* diaspores. The mean germination time is an important factor for the survival of weeds because plants that germinate more slowly may show reduced size, lower competition for resources, and less chance of establishment in the environment (Souza and others 2010; Grisi and others 2013). However, seedling development was more sensitive to the fractions than germination, and the principal reductions and morphological changes were observed for root growth. These results are in agreement with other authors who reported strong phytotoxic effects of allelochemicals on seedling growth in target species (Sodaeizadeh and others 2009; Ashrafi and others 2009; Souza and others 2010; Grisi and others 2012). Germinability, mean germination time, rate, and seedling length measurements (dependent variables) established cause-and-effect relationships with the concentration of the fractions tested (independent variable). Thus, it is possible to predict values for these characteristics at any concentration of the fractions within the studied interval (0–5.0 mg mL^{-1}).

In the present study, shoot elongation of *E. crus-galli* and *E. heterophylla* seedlings was more affected by the synthetic herbicide. However, the hexane and EtOAc fractions showed similar and superior inhibition, respectively, to the herbicide with regard to root length. The data obtained indicate that the root growth of both species was more responsive than the shoot growth to the inhibitory phytotoxic activity induced by the fractions. According to Grisi and others (2013), the oxyfluorfen herbicide acts with more intensity in the shoot, whereas the plant extracts invest in root growth inhibition. Abnormal seedling evaluation is also an important parameter in the detection of phytotoxic effects. The main anomalies identified for these weeds were root necrosis, a twisted or curved hypocotyl, the absence of lateral roots, stunted seedlings, and gravitropic inversion (Table 1). The results of the present study corroborate the findings of Ferreira and Áquila (2000), who reported that allelochemicals can induce the appearance of abnormal seedlings, in which necrosis is the most common symptom of the phytotoxic effect with the ability to inhibit the growth of the recipient plant.

The sensitivity of roots to allelochemicals is the characteristic that best indicates the allelopathic action of plant extracts. Previous studies (Souza and others 2010; Grisi and others 2012; Tanveer and others 2012) have also documented this aspect of a greater root than shoot inhibition. Possible reasons are because roots are the first to emerge and are in direct contact with extracts and thus are exposed to the peak periods and concentrations of phytotoxins (Tanveer and others 2012). Furthermore, the root is less protected by a cuticle than shoots/hypocotyls, which can result in greater penetration and concentration of these chemicals into root tissues (Yoshimura and others 2011). Root growth is characterized by high metabolic rates, and for this reason, roots are highly susceptible to environmental stress, such as allelochemicals in the substrate (Cruz-Ortega and others 1998). At the cellular level, allelochemicals induce lipid peroxidation, affect certain enzymatic activity, and rapidly depolarize the root cell membrane, resulting in a generalized increase in membrane permeability and thus blocking plant nutrient uptake (Santos and others 2008). Considering that the plant root system is its connection with the physical environment and the path of ingress of water and nutrients, poor root formation could affect the plant physiological state and thus prevent the establishment of weeds. Khanh and others (2005) reported that the highest level of allopathic suppression may occur when maximum levels of phytotoxins coincide with early stages of plant growth.

The reduction in the growth of *E. heterophylla* seedling roots treated with *S. saponaria* fractions might be

associated with inhibition of metaxylem cell elongation, suggesting probable interference of allelochemicals in the hormone balance (Gatti and others 2010). Recent studies have revealed that phytohormones play an important role in controlling cell division, cell growth, and cell differentiation in distinct zones of roots (Takatsuka and Umeda 2014). Auxin is an important long- and short-distance signal and controls multiple developmental processes, including root patterning (Friml and others 2002; Petersson and others 2009), cell division, and cell elongation in roots (Ding and Friml 2010). Polar auxin transport, which is mediated by PIN-FORMED (PIN) efflux carriers, is essential for creating auxin gradients and for proper development of organs (Tanaka and others 2006).

Using phytochemical analyses, Wahab and Selim (1985) detected the presence of flavonoids, lipids, and steroids in apolar extracts from *S. saponaria* seeds. Lemos and others (1992) isolated a new saponin from the ethyl acetate fraction of *S. saponaria* fruits and reported its anti-microbial activity. Murgu and Rodrigues-Filho (2006) reported that the main glycosides in the fruits of this species were saponins (SAP) derived from triterpenes hederagenin and oleanolic acid and oligoglycosides from acyclic sesquiterpene oligoglycosides (ASOGs). These authors detected up to 30 SAPs and 63 ASOGs, which the plant produces as a complex mixture of naturally acetylated glycosides. In the present study, 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid was identified in the EtOAc fraction obtained from *S. Saponaria* leaves. This metabolite is a monoterpene acid and has not been reported in the literature. Terpenes are widely found in the genus *Sapindus* (Pelegrini and others 2008; Huang and others 2008; Jeyabalan and Palayan 2009; Morikawa and others 2009; Eddaya and others 2013), with diverse biological activities and pharmacological properties, such as antidiabetic (Mahar and others 2011), antifungal (Tsuzuki and others 2007), and antibacterial (Lasisi and others 2012) activities.

The isolated 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid showed phytotoxic activity on the weeds studied. Only with regard to the germination of *E. crus-galli* and *E. heterophylla* diaspores did the isolated compound have a greater inhibition than the EtOAc fraction, whereas this fraction showed the highest inhibitory activity on seedling growth. Teerarak and others (2012) also observed that the inhibitory effect of odorine (active compound) on *E. crus-galli* germination and seedling growth decreased in comparison with the mixture of the crude EtOAc fraction. These results indicated that the EtOAc fraction may contain many active chemical constituents that may act synergistically. This finding is in agreement with the observation of synergistic of biological compounds reported by Kilani and others (2008) and Teerarak and others (2012). This approach will require further investigation to identify other biologically active metabolites.

The EtOAc fraction obtained from the ethanolic extract of *S. saponaria* leaves showed an inhibitory effect on seedling development that was greater than the hexane fraction. The compound 3-(1,2-dimethyl-5-oxabicyclo [2.1.1] hexan-2-yl) but-2-enoic acid isolated and identified from the EtOAc fraction also inhibited the germination and growth of *E. crus-galli* and *E. heterophylla*, but the EtOAc fraction was more potent in inhibiting seedling growth. Thus, the compounds present in the EtOAc fraction obtained from *S. saponaria* leaves can be potent weed growth regulators and offer promising alternatives for the sustainable control of these species.

Acknowledgments The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2011/11.860-5) for financial support.

References

Aliotta G, Cafiero G, Otero AM (2006) Weed germination, seedling growth and their lesson for allelopathy in agriculture. In: Reigosa MJ, Pedrol N, González L (eds) Allelopathy: a physiological process with ecological implications. Springer, Dordrecht, pp 285–297

Ashrafi ZY, Sadeghi S, Mashhadi HR (2009) Inhibitive effects of barley (*Hordeum vulgare*) on germination and growth of seedling quack grass (*Agropyrum repens*). Icel Agric Sci 22:37–43

Batish DR, Arora K, Singh HP, Kohli RK (2007) Potential utilization of dried powder of *Tagetes minuta* as a natural herbicide for managing rice weeds. Crop Prot 26:566–571

Brasil (2009) Ministério da Agricultura e Reforma Agrária. Coordenação de Laboratório Vegetal. Regras para análise de sementes, Brasília

Chou CH (1999) Roles of allelopathy in plant biodeversity and sustainable agriculture. Crit Rev Plant Sci 18:609–630

Cruz-Ortega R, Anaya AL, Hernández-Bautista BE, Laguna-Hernández G (1998) Effects of allelochemical stress produced by *Sicyos deppei* on seedling root ultrastructure of *Phaseolus vulgaris* and *Cucurbita ficifolia*. J Chem Ecol 24:2039–2057

Ding Z, Friml J (2010) Auxin regulates distal stem cell differentiation in *Arabidopsis* roots. Proc Natl Acad Sci USA 107:12046–12051

Dorning M, Cipollini D (2006) Leaf and root extracts of the invasive shrub, *Lonicera maackii*, inhibit seed germination of three herbs with no autotoxic effects. Plant Ecol 184:287–296

Eddaya T, Boughdad A, Sibille E, Chaimbault P, Zaid A, Amechrouq A (2013) Biological activity of *Sapindus mukorossi* Gaerten (Sapindaceae) aqueous extract against *Thysanoplusia orichalcea* (Lepidoptera: Noctuidae). Ind Crop Prod 50:325–332

Ferreira AG, Áquila MEA (2000) Alelopatia: uma área emergente da ecofisiologia. Rev Bras Fisiol Veg 12:175–204

Friml J, Benková E, Blilou I, Wisniewska J, Hamann T, Ljung K, Woody S, Sandberg G, Scheres B, Jürgens G, Palme K (2002) AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. Cell 108:661–673

Gatti AB, Ferreira AG, Arduin M, Perez SCJGA (2010) Allelopathic effects of aqueous extracts of *Aristolochia esperanzae* O. Kuntze on development of *Sesamum indicum* L. seedlings. Acta Bot Bras 24:454–461

Grisi PU, Ranal MA, Gualtieri SCJ, Santana DG (2012) Allelopathic potential of *Sapindus saponaria* L. leaves in the control of weeds. Acta Sci Agron 34:1–9

Grisi PU, Gualtieri SCJ, Anese S, Pereira VC, Forim MR (2013) Effect of *Serjania lethalis* ethanolic extract on weed control. Planta Daninha 31:239–248

He HQ, Shen LH, Xiong J, Jia XL, Lin WX, Wu H (2004) Conditional genetic effect of allelopathy in rice (*Oryza sativa* L.) under different environmental conditions. Plant Growth Regul 44:211–218

Hong NH, Xuan TD, Tsuzuki E, Terao H, Matsuo M, Khanh TD (2004) Weed control of four higher plant species in paddy rice fields in Southeast Asia. J Agron Crop Sci 190:59–64

Huang HC, Wu MD, Tsai WJ, Liao SC, Liaw CC, Hsu LC, Wu YC, Kuo YH (2008) Triterpenoid saponins from the fruits and galls of *Sapindus mukorossi*. Phytochemistry 69:1609–1616

Jeyabalan S, Palayan M (2009) Antihyperglycemic and antidiabetic activity of leaves extracts of *Sapindus emarginatus* Vahl. Asian Biomed 3:313–318

Khan M, Hussain F, Musharaf S, Imdadullah (2011) Allelopathic effects of *Rhazya stricta* decne on seed germination and seedling growth of maize. Afr J Agr Res 6:6391–6396

Khanh TD, Chung MI, Xuan TD, Twata S (2005) The exploitation of crop allelopathy in sustainable agricultural production. J Agro Crop Sci 191:172–184

Kilani S, Sghaier MB, Limem I, Bouhlel I, Boubaker J, Bhouri W, Skandrani I, Neffatti A, Ammar RB, Dijoux-Franca MG, Ghedtra K, Chekir-Ghedira L (2008) In vitro evaluation of antibacterial, antioxidant, cytotoxic and apoptotic activities of the tubers infusion and extracts of *Cyperus rotundus*. Bioresour Technol 99:9004–9008

Kobayashi K (2004) Factors affecting phytotoxic activity of allelochemicals in soil. Weed Biol Manag 4:1–7

Köppen W (1948) Climatologia: com um estúdio de los climas de la tierra. Fondo de Cultura Econômica, México

Kraus JE, Arduin M (1997) Manual básico de métodos em morfologia vegetal. Seropedica, EDUR

Lasisi AA, Ayinde BW, Adeleye AO, Onocha PA, Oladosu IA, Idowu PA (2012) New triterpene isovanniloyl and antibacterial activity of constituents from the roots of *Paullinia pinnata* Linn (Sapindaceae). J Saudi Chem Soc. doi:10.1016/j.jscs.2011.12.012

Lemos TLG, Mendes AL, Sousa MP, BrazFilho R (1992) New saponin from *Sapindus saponaria*. Fitoterapia 63:515–517

Macías FA, Lacret R, Varela RM, Nogueiras C, Molinillo JMG (2010) Isolation and phtytotoxicity of terpenes from *Tectona grandis*. J Chem Ecol 36:396–404

Mahar KS, Rana TS, Ranade SA, Meena B (2011) Genetic variability and population structure in *Sapindus emarginatus* Vahl from India. Gene 485:32–39

Marco CA, Teixeira E, Simplício A, Oliveira C, Costa J, Feitosa J (2012) Chemical composition and allelopathyc activity of essential oil of *Lippia sidoides* Cham. Chilean J Agric Res 72:157–160

Morikawa T, Xie Y, Asao Y, Okamoto M, Yamashita C, Muraoka O, Matsuda H, Pongpiriyadacha Y, Yuan D, Yoshikawa M (2009) Oleanane-type triterpene oligoglycosides with pancreatic lipase inhibitory activity from the pericarps of *Sapindus rarak*. Phytochemistry 70:1166–1172

Murgu M, Rodrigues-Filho E (2006) Dereplication of Glycosides from *Sapindus saponaria* using Liquid Chromatography-Mass Spectrometry. J Brazil Chem Soc 17:1281–1290

Panozzo LE, Agostinetto D, Moraes PVD, Magano DA, Harter A, Pinto LB (2014) Control of *Echinochloa* sp. in the Irrigated Rice Crop. Int J Agron 2014:1–6

Parvez SS, Parvez MM, Nishihara E, Gemma H, Fujii Y (2003) *Tamarindus indica* L. leaf is a source of allelopathic substance. Plant Growth Regul 40:107–115

Pelegrini DD, Tsuzuki JK, Amado CAB, Cortez DAG, Ferreira ICP (2008) Biological activity and isolated compounds in *Sapindus saponaria* L. and other plants of the genus *Sapindus*. Lat Am J Pharm 27:922–927

Petersson SV, Johansson AI, Kowalczyk M, Makoveychuk A, Wang JY, Moritz T, Grebe M, Benfey PN, Sandberg G, Ljung K (2009) An auxin gradient and maximum in the *Arabidopsis* root apex shown by highresolution cell-specific analysis of IAA distribution and synthesis. Plant Cell 21:1659–1668

Pimentel-Gomes FP (1990) Curso de estatística experimental, 13th edn. Nobel, Piracicaba

Prati D, Bossdorf O (2004) Allelopathic inhibition of germination by *Alliaria petiolata* (Brassicaceae). Am J Bot 91:285–288

Ranal MA, Santana DG (2006) How and why to measure the germination process? Rev Bras Bot 29:1–11

Rodrigues BN, Almeida FS (2011) Guia de herbicidas, 6th edn. Edição dos Autores, Londrina

Sampietro DA, Vattuone MA (2006) Sugarcane straw and its phytochemicals as growth regulators of weed and crop plants. Plant Growth Regul 48:21–27

Santos WD, Ferrarese ML, Nakamura CV, Mourão KS, Mangolin CA, Ferrarese-Filho O (2008) Soybean (*Glycine max*) root lignification induced by ferulic acid. The possible mode of action. J Chem Ecol 34:1230–1241

Sodaeizadeh H, Rafieiolhossaini M, Havlik J, Damme PV (2009) Allelopathic activity of different plant parts of *Peganum harmala* L. and identification of their growth inhibitors substances. Plant Growth Regul 59:227–236

Souza FM, Gandolfi S, Perez SCJGA, Rodrigues RR (2010) Allelopathic potential of bark and leaves of *Esenbeckia leiocarpa* Engl. (Rutaceae). Acta Bot Bras 24:169–174

Takatsuka H, Umeda M (2014) Hormonal control of cell division and elongation along differentiation trajectories in roots. J Exp Bot 65:2633–2643

Tanaka H, Dhonukshe P, Brewer PB, Friml J (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell Mol Life Sci 63:2738–2754

Tanveer A, Jabbar MK, Kahliq A, Matloob A, Abbas RN, Javaid MM (2012) Allelopathic effects of aqueous and organic fractions of *Euphorbia dracunculoides* Lam. on germination and seedling growth of chickpea and wheat. Chil J Agric Res 72:495–501

Tawaha AM, Turk MA (2003) Allelopathic effects of Black Mustard (*Brassica nigra*) on germination and growth of Wild Barley (*Hordeum spontaneum*). J Agron Crop Sci 189:298–303

Teerarak M, Charoenying P, Laosinwattana C (2012) Physiological and cellular mechanisms of natural herbicide resource from *Aglaia odorata* Lour. on bioassay plants. Acta Physiol Plant 34:1277–1285

Tsuzuki JK, Svidzinski TI, Shinobu CS, Silva LF, Rodrigues-Filho E, Cortez DA, Ferreira IC (2007) Antifungal activity of the extracts and saponins from *Sapindus saponaria* L. An Acad Bras Cienc 79:577–583

Vidal RA, Trezzi MM, Prado R, Ruiz-Santaella JP, Vila-Aiub M (2007) Glyphosate resistant biotypes of wild poinsettia (*Euphorbia heterophylla* L.) and its risk analysis on glyphosate-tolerant soybeans. J Food Agric Environ 5:265–269

Wahab SMA, Selim MA (1985) Lipids and flavonoids of *Sapindus saponaria*. Fitoterapia 56:167–168

Wink M (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64:3–19

Xuan TD, Shinkichi T, Khanh TD, Chung IM (2005) Biological control of weeds and plant pathogens in paddy rice by exploiting plant allelopathy: an overview. Crop Prot 24:197–206

Yoshimura H, Sawa Y, Tamotsu S, Sakai A (2011) 1,8-cineole inhibits both proliferation and elongation of by-2 cultured tobacco cells. J Chem Ecol 37:320–328