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Phytotoxicity of *Quillaja brasiliensis* leaf saponins on *Lactuca sativa* and *Echinochloa crus-galli* and their potential as a bioherbicide

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Phytotoxicity of *Quillaja brasiliensis* leaf saponins on *Lactuca sativa* and *Echinochloa crus-galli* and their potential as a bioherbicide

Abstract

The challenge of food security to meet the needs of an increasing human population demands the adoption of new approaches leading to sustainable and sufficient crop production. A high percentage of crop products are lost every year due to agricultural pests. Weeds are the most damaging class of pests and threaten the integrity of agricultural and natural environments due to their invasive and competing potential. Pesticide use in agriculture has increased in recent years and although it increases agricultural production, it may cause undesired negative environmental impacts. Bioherbicides, which can be used as weed management tools, consist of substances based on natural compounds already present in the environment being biodegradable and having low residual effects. The use of plant species able to produce and release phytotoxic compounds may represent effective bioherbicide sources. *Quillaja brasiliensis* produces secondary metabolites called saponins that could be evaluated for phytotoxic activity and potentially become a natural herbicide. Therefore, this study was conducted to examine the phytotoxic activity of *Q. brasiliensis* saponins aqueous extract (AE) and saponin fraction (QB) on morpho-physiological parameters of *Lactuca sativa* (lettuce) and *Echinochloa crus-galli* (barnyardgrass), in pre and post-emergence bioassays. In the pre-emergence bioassays, germination rate and speed of germination were determined. The seedlings of the same species were evaluated regarding effects on initial growth by measuring seedling root and shoot length, dry mass and chlorophyll content. Both aqueous extract and saponin fraction had high inhibitory impact on germination of lettuce and barnyardgrass. Osmotic potential analyses revealed that this parameter was not important in the observed responses. Saponin fraction at 1% and 2% (w/v) concentration significantly decreased shoot length of lettuce seedlings by more than 10-fold. Results also showed a phytotoxic effect on post-emergence growth of lettuce, especially at the highest concentration tested of AE (10% w/v). These results show that both saponin enriched fraction and aqueous extracts of *Q. brasiliensis* are phytotoxic. Further studies should aim at detailing their phytotoxic mechanism on plants aiming at their possible use as bioherbicides.

Keywords: *Quillaja brasiliensis*, Phytotoxic plant extract, Saponin, Bioherbicide.

1. Introduction

The combination of efficient agricultural land use with biodiversity conservation is a challenge (Tscharntke et al., 2012). According to a 2017 United Nations report, the world's population is projected to reach 9.8 billion by 2050, with 83 million people being added every year. With that comes a rising demand for food at a global level. Data indicates that agriculture will need to provide almost 50 percent more food in 2050 (FAO 2018). In order to sustain this level of production and knowing that 20–40% of global crop yields are lost each year to pests and diseases, pesticide use in agriculture has increased in recent years (FAO 2018). Due to its extensive planting area, Brazil is one of the three largest consumers of pesticides in the world (Pignati, 2017; FAO 2018).

"Pests" are the considered invasive alien organisms or pathogens of crops or forest species (Bebber et al. 2014) and include animal pests (insects, mites, nematodes, rodents, slugs and snails, birds), plant pathogens (viruses, bacteria, fungi, chromists) and weeds (i.e. competitive plants) (Oerke, 2006). Weeds are the most damaging category of pests in agriculture (Stewart, 2017), potentially reducing the production of crops by 34%, followed by animal pests (18%) and pathogens (16%) (Oerke, 2006).

A particular plant is considered a "weed" only in terms of anthropocentric definitions (Zimdahl, 2018). Schonbeck (2011) defines weed as a “plant out of place,” an “unwanted plant” or a plant that interferes with crop or livestock production. The definition by the Weed Science Society of America (2016) states: a weed is a plant that causes economic losses or ecological damage, creates health problems for humans or animals, or is undesirable where it is growing. Most agricultural weeds cause damage to crop yields or increase costs of production due to a number of features, including: a) capacity to grow in disturbed habitats, b) show high environmental plasticity (many weeds are capable of tolerating and growing under a wide range of climatic and edaphic conditions), c) ability to physically hinder or smother crop growth, d) capacity to compete with crops for light, nutrients, moisture, and space, e) have rapid emergence, seedling growth and quick maturation, f) produce a large number of seeds per plant, g) release natural substances that inhibit crop growth (allelopathy), h) host pests or pathogens that may contaminate crops (Zimdahl, 2018; Schonbeck, 2011).

Pesticides are toxic chemical substances or a mixture of substances that are intentionally released into the environment in order to control or eliminate pests (Hakeem et al., 2016). Although pesticides prevent, destroy and repel most pests growing on crop plants,

their continued use can cause significant impact on the environment and human health by contaminating waterbodies, air, terrestrial ecosystems and produce, as well as unintentionally affect biodiversity (Caldas, 2016, Abbas et al., 2018; Tirado et al., 2008). This occurs especially in low and middle-income countries where government policies tend to be less adequate and health surveillance less effective (Carneiro et al., 2015). Thus, there is an increasing demand for pesticides that are safe for the environment and may replace or complement synthetic managing methods (Cordeau et al., 2016). Weed management techniques can vary (manual, mechanical, chemical, and biological control), but mostly depend on the use of synthetic herbicides (pre-emergent and post-emergent) (Abbas et al., 2018; Chauhan and Mahajan, 2014). Hence, it is important to search for new and effective alternatives for weed management. Furthermore, in Brazil, herbicides represent the highest percentage class of imported pesticides (58.45%) and are used in far larger volumes than insecticides and fungicides combined (IBAMA, 2016).

It has been proven that sustainable practices reduce agriculture's negative environmental impacts, decrease production costs, minimize collateral damage to wildlife and minimize the social pressure from the public about food safety and human security (Cordeau et al., 2016). The use of biological control, including bioherbicides, consists on the application of living organisms (insects, nematodes, bacteria, or fungi), natural products or biotic agents to mitigate weeds in agriculture or natural ecosystems reducing environmental impact (Cai and Gu, 2016; Kremer, 2019). Substances obtained from living organisms e.g. the natural metabolites produced during growth and development can be ingredients of bioherbicides; although the uses of these metabolites are highly efficient and beneficial, only 8% of conventional herbicides are derived from natural compounds (Dayan and Duke, 2014). Accordingly, the use of plant species able to produce and release phytotoxic compounds may represent an effective tool to be used alone or in association with other plant protection methods for weed management (Cordeau et al., 2016; Puig et al., 2018).

Natural phytotoxins, their structural diversity and varied biological activity offer several benefits when compared to synthetic compounds. They tend to have a shorter half-life, are rich in bioactive materials and compounds with unexploited properties and are produced by different plant species (Duke et al., 2000; Duke et al., 2002). Saponins are secondary metabolites with complex chemical structure and high variability among organisms, which apparently are involved in environmental adaptation of plants (De Costa et

al., 2014; Nascimento and Fett-Neto, 2010). These molecules have a wide distribution among plants and several significant industrial and pharmacological applications (Güçlü-Üstündağ and Mazza, 2007; Yendo et al., 2016). Several studies have revealed that saponins have phytotoxic or allelopathic properties (Faizal and Geelen, 2013; Hoagland et al., 1996; Jelassi et al., 2016; Pérez et al., 2015; Ribeiro et al., 2018; Stavropoulou et al., 2017; Wyman-Simpson et al., 1991), which suggests that saponins could be evaluated as potential bioherbicides (Dayan and Duke, 2014).

Quillaja brasiliensis (A. St.-Hill. & Tul.) Mart. (Quillajaceae) (soap tree) is distributed throughout Southern Brazil (Reitz, 1996). The species is well known for the abundant saponin presence and therefore large spectrum of biological activities, including immunoadjuvant, antifungal and antiherbivore (Fleck et al., 2006; Silveira et al., 2011; De Costa et al., 2014; Cibulski et al., 2016; Anna Yendo, Plant Physiology Laboratory, UFRGS, personal communication). According to Kauffmann et al. (2004), the structure of *Q. brasiliensis* saponins is remarkably similar to that of *Quillaja saponaria* Molina bark saponins. The latter is a related Chilean species which has been widely used as an adjuvant in vaccine formulations and is one of the main sources of industrial saponins present in plants. The commercially used saponin fraction obtained from barks of *Q. saponaria* is known as Quil-A® (Yendo et al., 2015). Studies have reported *Q. saponaria* as showing strong aphicidal, deterrent, nematicidal, molluscicidal and antifungal activity, indicating that the plant could be potentially used as a biopesticide (González-Cruz and Martín, 2013; De Geyter et al., 2011; Moya et al., 2010; González-Castillo et al., 2018; Giannakou, 2011). Anti-fungal, molluscicide, and insecticide activities have also been shown for leaf saponins of *Q. brasiliensis* (Anna Yendo, Center for Biotechnology-UFRGS, personal communication). If *Q. brasiliensis* saponins prove to be phytotoxic, there is also a potential use of these natural products as bioherbicides. In addition, the use as a biopesticide can be explored as a combination with other molecules at lower concentrations, thereby preventing resistance development. Regardless of the potential use as a biopesticide or bioherbicide, *Q. brasiliensis* leaf saponins consist of a more sustainable and easily renewable alternative if compared to *Q. saponaria* bark saponins, considering that the second has a destructive effect of phloem stripping of trees during bark removal and depends on slow growing native forests (Martín and Briones, 1999). Hence, this study was conducted to examine the phytotoxic activity of

aqueous extracts and saponins of *Q. brasiliensis* on different morpho-physiological parameters of target species using *in vitro* bioassays.

2. Materials and methods

2.1. Plant material and extracts preparations

Q. brasiliensis leaves were collected from adult plants growing in the city of Canguçu, RS, Brazil (31º23'42''S-52º40'32''W). A voucher specimen was deposited at the ICN Herbarium of the Federal University of Rio Grande do Sul (142953). Air-dried powdered leaves were extracted in distilled water (1:10, w/v) for 8 h, filtered, partitioned with ethyl acetate and lyophilized, yielding aqueous extract (AE). AE was submitted to purification through reverse-phase chromatography and gradient of water and methanol to obtain fraction QB-90, as previously described (Fleck et al., 2006). QB-80 was obtained using the same protocol. QB-80 and QB-90 were also analyzed by TLC to further confirm fraction isolation.

2.2. Phytotoxicity assay

2.2.1. Plant material

The experiments on the phytotoxic effect of the extract were carried out with diaspores (herein referred to as seeds) of the standard target species *Lactuca sativa*, as well as seedlings of the same species; and with seeds and seedlings of the weed species *Echinochloa crus-galli*. For these assays, commercially available lettuce seeds were used (Isla®, lote 116377-001) and barnyardgrass seeds were kindly provided by the Department of Crop Plants – Faculty of Agronomy, UFRGS.

2.2.2. Establishment of osmotic potential and pH of the extract

Both osmotic potential and pH of the extracts were determined. The osmotic potential of *Q. brasiliensis* extracts were measured in different concentrations (0.1%, 1%, 2%, 4%, 10%, 20%, 40%) with a refractometer-based curve prepared with sucrose. To determine the influence of the osmotic potential of the extracts on both lettuce and barnyardgrass germination bioassays, additional tests were performed with solutions of polyethylene glycol (PEG-6000) in the following concentrations: 0.01 M, 0.02 M and 0.03 M. This experiment was carried out using the same procedure of germination bioassays. The osmotic potential of

the different concentrations of PEG was also measured with a refractometer and it was possible to associate the solution of PEG at 0.02 M with the 10% *Q. brasiliensis* extract. Extracts pH were determined with a pH meter.

2.2.3. Pre-emergence bioassay

The pre-emergence bioassay analyzed the germination of *L. sativa* (lettuce) and *Echinochloa crus-galli* (barnyardgrass). Petri dishes (9 cm of diameter) lined with qualitative filter paper were used and assays were conducted in a growth chamber BOD (Biological Oxygen Demand) (25º C, 12 h/12 h dark/light, 40 µmol photons.m^{-2}.s^{-1}).

For each one of the species, the petri dishes were separated in experimental groups and control, each containing 25 seeds per plate, with 3 plates per experimental group. Seeds were treated with 5 mL in each petri dish of the different extracts of *Q. brasiliensis* (at 4 and 10% w/v of the extract), distilled water (negative control), and NaCl 0.5 M (positive control). Germination parameters were measured daily (germination time) or on the 5th day of incubation (germination percentage, root and shoot length). The presence of morphological abnormalities was recorded. Germination speed index were calculated according to Maguire (1962):

$$IVG = N1 / D1 + N2 / D2 + ... + Nn / Dn$$

Where: IVG = germination velocity index; N =number of seedlings at the day of counting; D = days after sowing until data collection.

An additional experiment using a purified saponin fraction called QB was performed with lettuce. The same parameters were analyzed, with treatments consisting of 1 and 2% of QB-80 and -90 and controls, in this case ending on the 7th day of incubation.

2.2.4. Bioassay for post-emergence

To obtain lettuce seedlings for the effect on initial growth, seeds were put in separate pot trays (each with a volume of 15 mL) containing autoclaved substrate (commercial previously washed sand: vermiculite - ratio 1:1; v/v) and kept for 12 days in a room with controlled light and temperature (photoperiod of 16 h per day; 25 ± 2°C, irradiance 50 µmol.m-^{2}.s^{-1}). On the 12th day, plants were exposed to the different extracts and controls (2

mL per pot containing a single plant and sprinkling to dew point). Barnyardgrass seeds were kept for 13 days in the substrate in the same conditions above and on the 14th day the seedlings were exposed to the treatments.

Seven days after treatment application, the growth parameters were measured (radicle length, shoot length and dry mass). Root and shoot length were measured with ruler and dry mass at 60°C was recorded in an analytical balance. In addition, for lettuce, the chlorophyll content was measured, with a SPAD chlorophyllometer (Minolta). With barnyardgrass, due to restrictions of leaf size on the SPAD reading probe, standard spectrophotometric evaluation of acetone extracts was carried out (Ross, 1974). All treatments for each sampling time (1 plant per separate slot) were replicated 50 times for root length, shoot length, dry mass measurements and SPAD readings and 20 times (samples of 5 plants) for spectrophotometric chlorophyll measurements.

In another set of experiments, *Q. brasiliensis* extracts and control treatments were applied on post-emergent 12-day-old lettuce seedlings grown in petri dishes. After 12 days of growth in BOD conditions, 5 mL of extracts and controls were applied on each dish containing 25 plants, replicated 3 times. Plants were measured after 7 days of application (root length, shoot length and dry mass).

2.2.5. Soil leaching bioassay

In order to evaluate if the extract could be leached from the soil, two different bioassays were performed. With *L. sativa* 50 separate pots containing autoclaved substrate (commercial previously washed sand: vermiculite - ratio 1:1; v/v) were exposed to 2mL of the extracts plus spraying of 30mL at the concentrations of 4 and 10%. These pots were subjected to leaching with 10 mL of distilled water each pot every 2 days, for a total of 8 days. In the day of the last water application, 2 lettuce seeds were added per pot to evaluate germination and seedling growth at the end of 12 days, with the same parameters used in the post-emergence bioassay. The experiment with barnyardgrass evaluated the leaching potential with seedlings grown in the same substrate previously described for 12 days, with the application of the extracts and controls at the end of the 13th day. After 7 days of application, 10 mL of distilled water were applied in each separate plate to leachate the extract away, every 2 days, 4 times. Same parameters were measured at the end of the last water application.

2.2.6. Statistical analyses

GraphPad Prism 5.0 was used to draw graphs. The results were analyzed by ANOVA followed by the Tukey test, when appropriate. The data was expressed as mean ± standard deviation (S.D.).

3. Results

3.1. Phytotoxicity assay

3.1.1. Osmotic potential and pH of extracts

The results obtained in the germination bioassay with PEG showed that solutions up to 0.02 M did not affect significantly the emergence of lettuce and barnyardgrass seeds. Since the 0.02 M solution was the equivalent to the 10% *Q. brasiliensis* extract, it was determined that up until 10%, the effects produced by the extract on the seeds were not due to the any osmotic effects of the extract (Table 1). The osmotic potential was determined following data of Meneses et al., 2007. Similarly, pH of the extract was in the 4-6 range, which is not capable of inhibiting germination of the test species (Sadeghloo et al., 2013; Rice, 1984).

Table 1. Density measured with a refractometer and the respective osmotic potential values of different concentrations of *Quillaja brasiliensis* aqueous extract (at 1%, 2%, 4%, 10%, 20% and 40%) and of the Polyethylene glycol (PEG-6000) (at 0.01 M, 0.02 M and 0.03 M).

	Density (g/mL)	Osmotic potential (MPa)
AE 1%	1.000	-0.191
AE 2%	1.000	-0.191
AE 4%	1.001	-0.192
AE 10%	1.034	-0.200
AE 20%	1.065	-0.205
AE 40%	>1.120	>-0.215
PEG 0.01 M	1.001	-0.192
PEG 0.02 M	1.042	-0.200
PEG 0.03 M	>1.120	>-0.215

3.1.2. Pre-emergence Bioassay

Both 4% and 10% extracts caused strong inhibitory effects on the emergence of seeds. For lettuce, no emergence was reported after 5 days, while the negative control had 97% of germination (Fig. 1A). Barnyardgrass had 16% of germination in the 4% AE and 75% in the negative control; the 10% extract inhibited all of the seeds germination (Fig. 2A).

The experiment using isolated saponins (QB fraction) with lettuce showed a significant reduction in the root and shoot length of the seedlings that germinated (Fig. 3) and the emergence declined with the increase in amount of QB concentration. At 1% QB the emergence of seeds was reduced by 75% while at 2% QB there was a reduction of 66% of germination (Fig. 1B).

The germination speed was also calculated and agreed the data of germination (Table 2). The extracts affected not only the percentage of germination but also the speed of the process in both species.

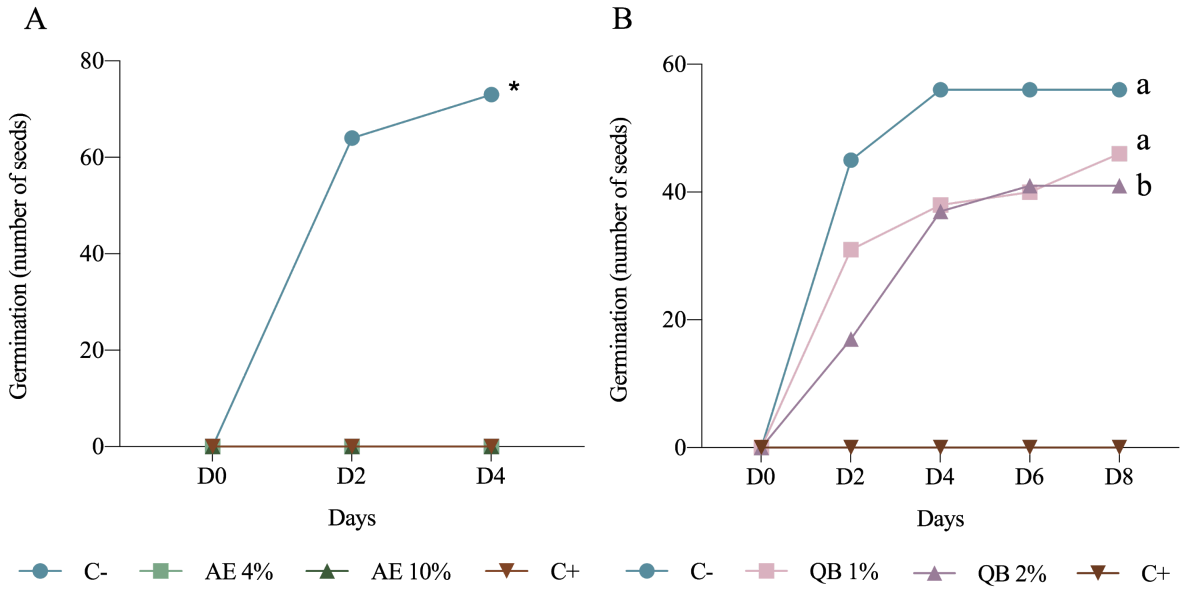


Fig. 1. Pre-emergence bioassay. Effects of (A) the aqueous extracts (at 4 and 10%) and (B) QB fractions (at 1 and 2%) on the number of germinated seeds of *L. sativa* at the end of 4 days and 8 days, respectively. C- represents the negative control (distilled water) and C+ the positive control (NaCl). Different letters and * indicate a significant difference by Tukey test ($p \leq 0.05$).

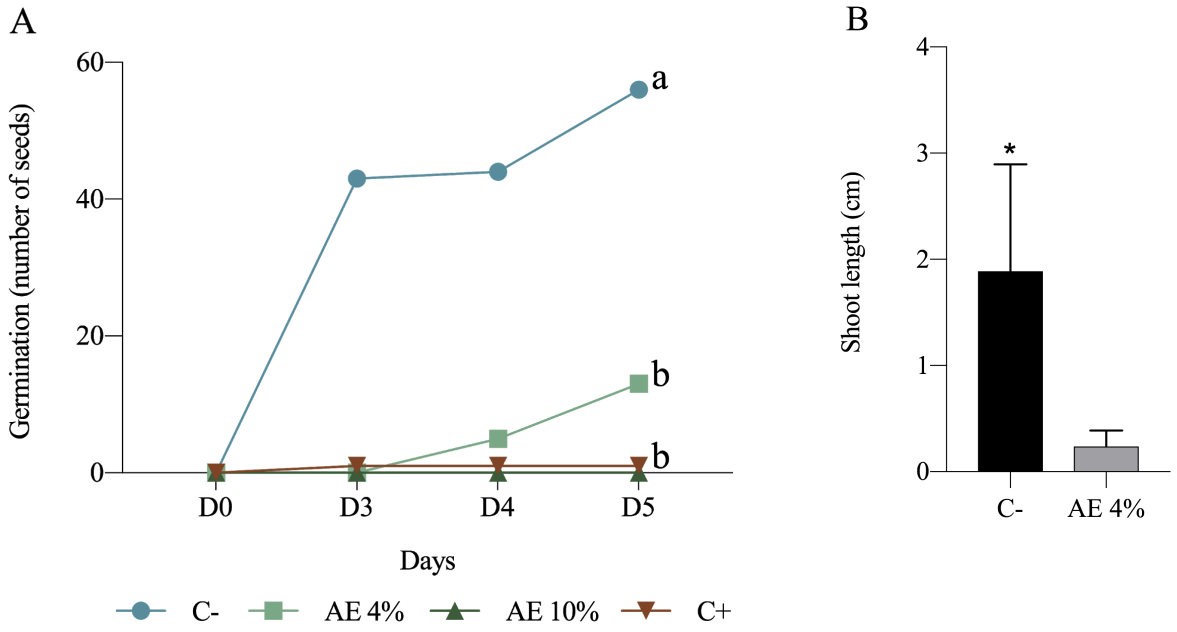


Fig. 2. Pre-emergence bioassay. Germination (A) in the different treatments (distilled water, aqueous extract at 4%, aqueous extract at 10% and NaCl) and seedling growth of shoot length at the end of 5 days (B) of barnyardgrass seeds. Only the 4% extract showed a few emerged seeds. In (B), bars represent the mean+SE. Different letters and * indicate a significant difference by Tukey test ($p \leq 0.05$).

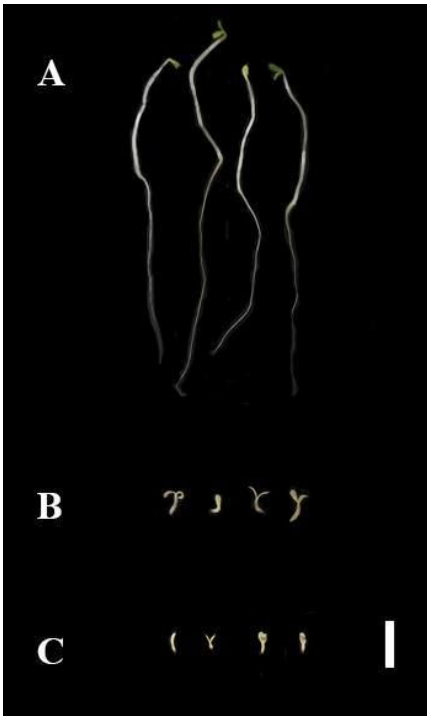


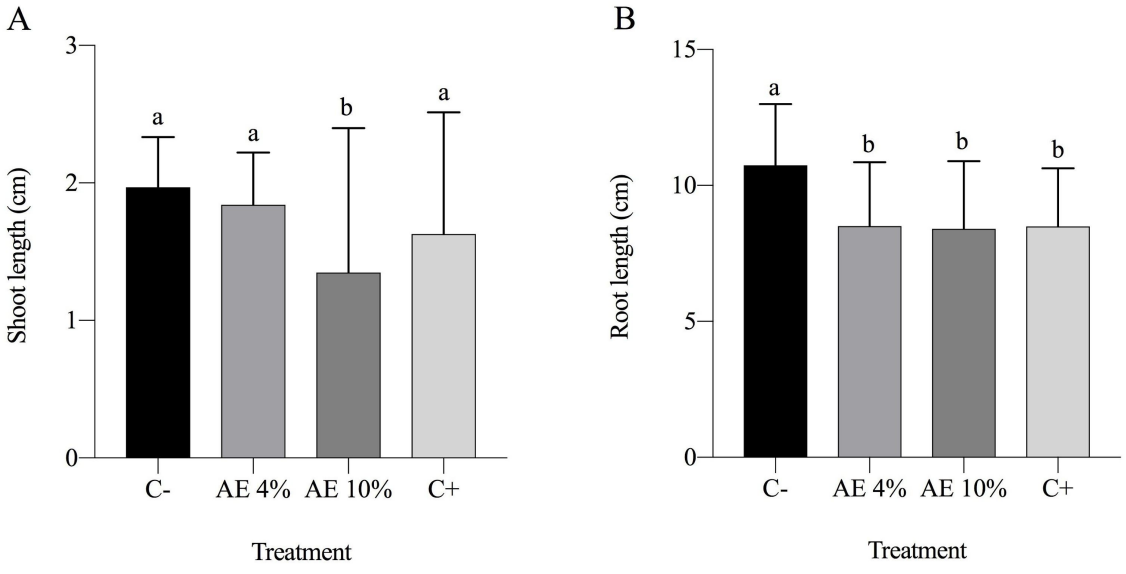
Fig. 3. Pre-emergence bioassay. Seedings of lettuce at the end of 8 days of application of (A) distilled water, (B) QB 1% and (C) QB 2%. Bar=1 cm.

Table 2. Germination speed index during 4 days (lettuce) and 5 days (barnyardgrass). Seeds were treated with the following treatments: extracts of *Q. brasiliensis* at 4% and 10%, distilled water (negative control), and NaCl 0.5 M (positive control).

	(W) lettuce	(W) barnyardgrass
C-	50,25	52,83
AE 4%	0	4,91
AE 10%	0	0
C+	0	1,08

3.1.3. Post-emergence bioassay

Seven days after the application of the treatments on lettuce seedlings, both 4 and 10% extract showed significant differences from the negative control in every parameter analyzed, except for the shoot length and dry weight in the lower concentration (4%) (Fig. 4). Interestingly, the chlorophyll content of the 10% extract decreased more than the one shown in the positive control (NaCl) (fig. 4C) and the plants from the 4% extract treatment showed abnormalities and low chlorophyll content in leaves (Fig. 5). Moreover, most of the seedlings from that treatment were notably fragile and the extract provoked the death of 24% of the plants, while the positive control only led to 16% of seedling viability loss (Table 3).



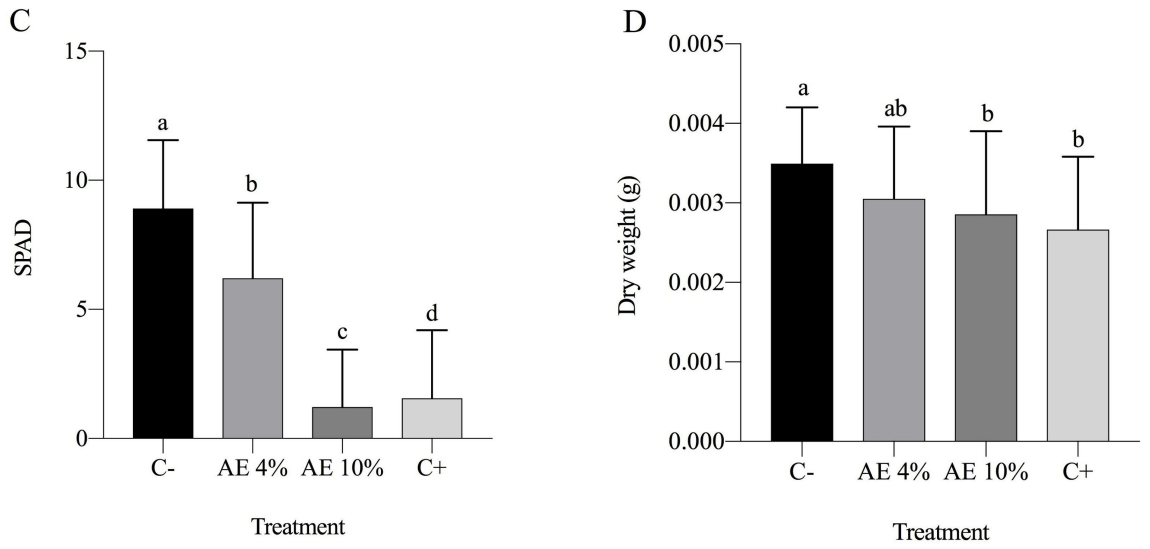


Fig. 4. Morphological parameters of lettuce seedlings in the post-emergence experiment. Plants were treated with distilled water (C-), aqueous extract at 4 and 10% (AE 4% and AE 10%) and NaCl (C+). In this bioassay, we analyzed the (A) shoot length; (B) root length; (C) chlorophyll content; and (D) dry mass. Bars represent the mean+SE. Different letters indicate a significant difference by Tukey test ($p \leq 0.05$).

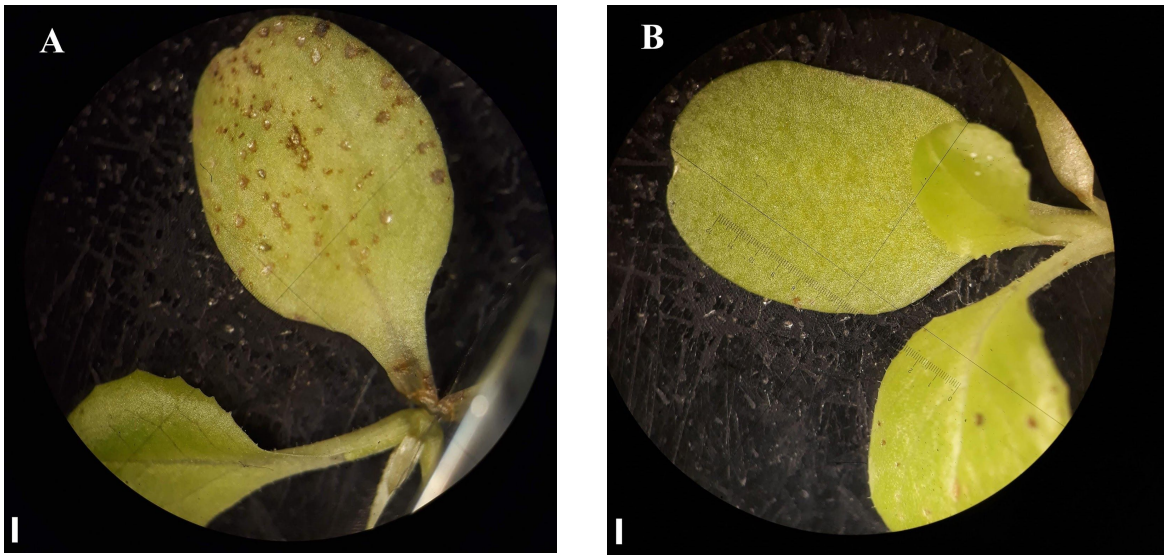


Fig. 5. *L. sativa* leaves at day 7 in the post-emergence bioassay, treated with (A) 4% extract and (B) the negative control (distilled water). Bar=1mm.

Table 3. Viability loss of seedlings at the end of the Post-emergence bioassay (7 days after application of treatments).

	Number of dead seedlings
C-	0
AE 4%	0
AE 10%	12 (24%)
C+	8 (16%)

With barnyardgrass, no significant difference was observed in root length, shoot length, chlorophyll content and dry weight between the treatments and compared to controls (Fig. 6). However, the plants from the 10% AE were wilty (Fig. 7).

In the additional post-emergence bioassay growing lettuce in petri dishes, a significant reduction was observed in the root and shoot elongation of the seedlings in both extract treatments (4% and 10%) and positive control (NaCl) in the same manner (Fig. 8). No significant difference was observed in the dry mass (Fig. 8C). The chlorophyll content wasn't measured in this experiment because the plants were too small.

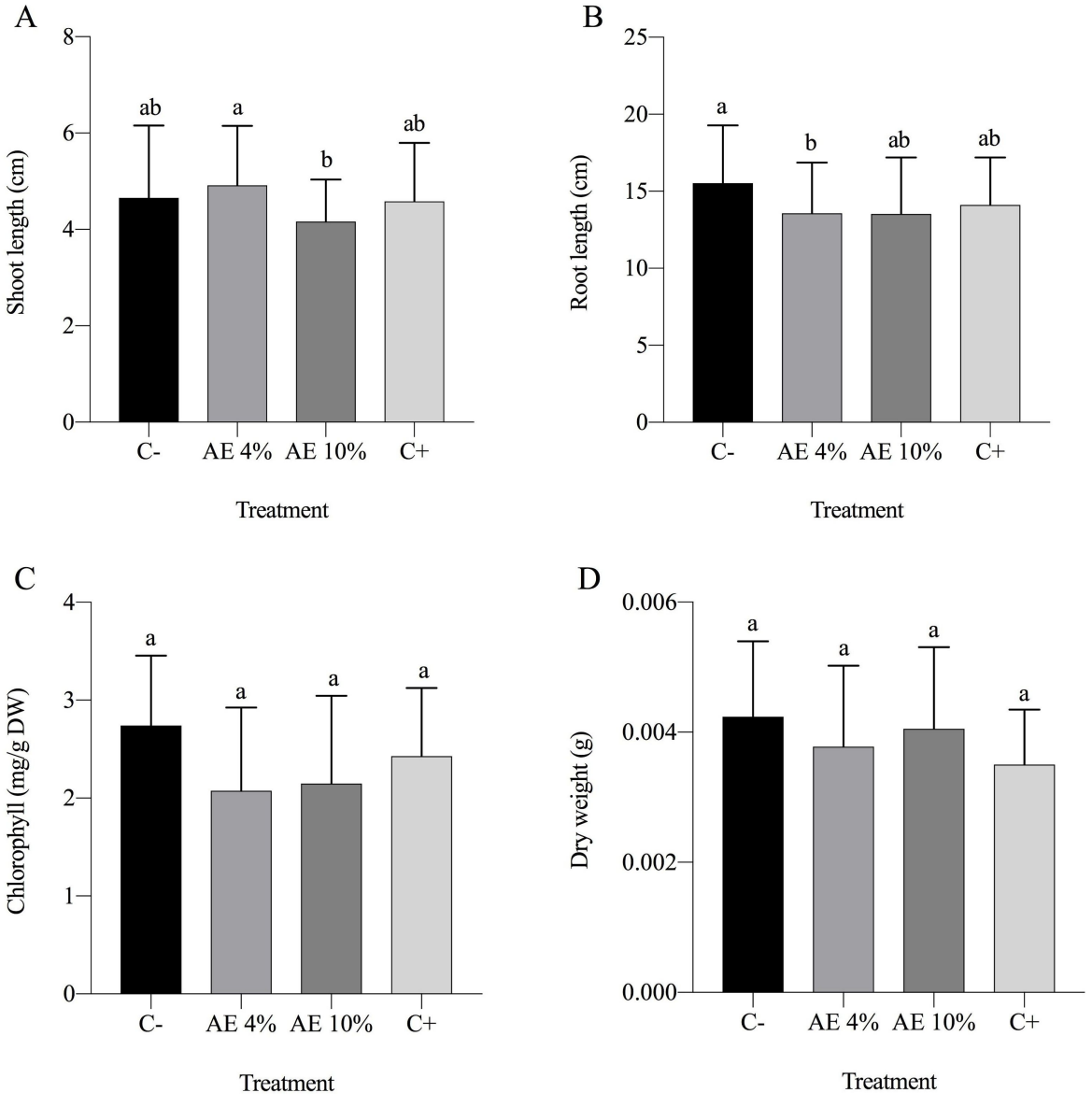


Fig. 6. Post-emergence bioassay with barnyardgrass. Morphological parameters: shoot length (A) and root length (B), chlorophyll content (C) and dry weight (D). Bars represent the mean+SE. Different letters indicate a significant difference by Tukey test ($p \leq 0.05$).

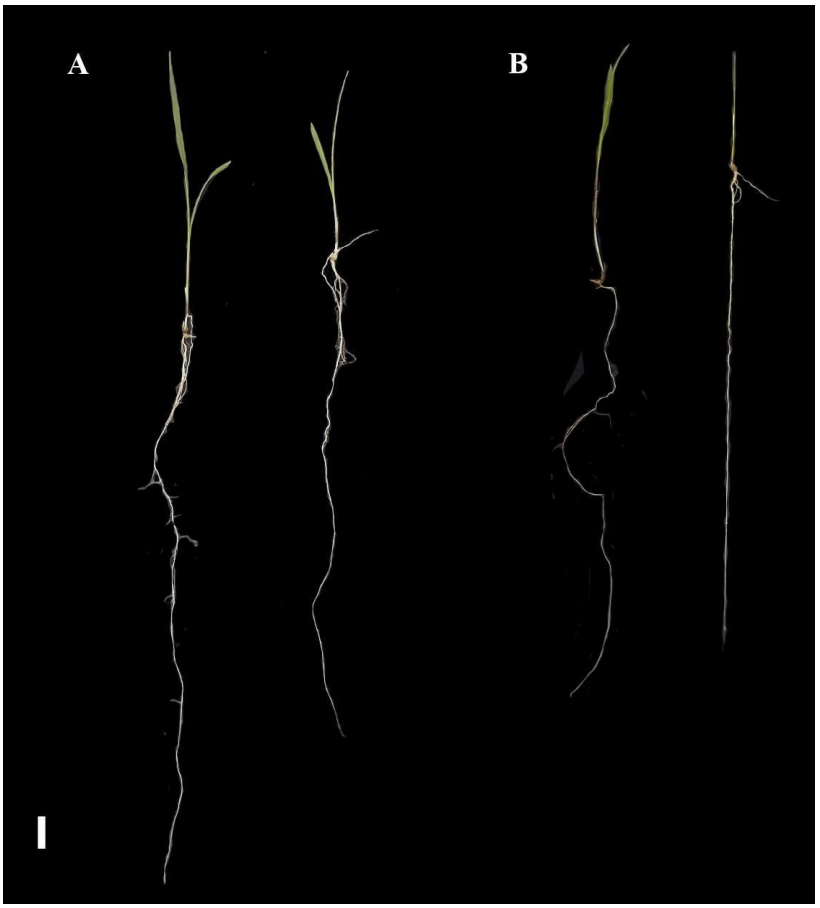
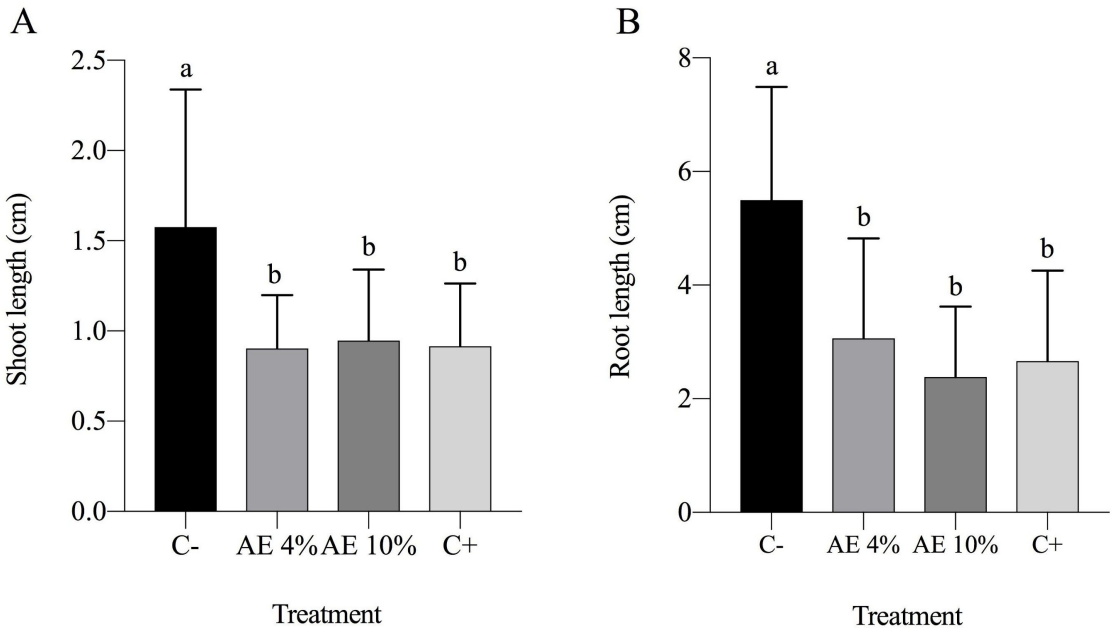


Fig. 7. Post-emergence bioassay with barnyardgrass. Seedlings at the end of 7 days treated with (A) distilled water – C- and (B) aqueous extract at 10% – AE 10%. Bar=1 cm.



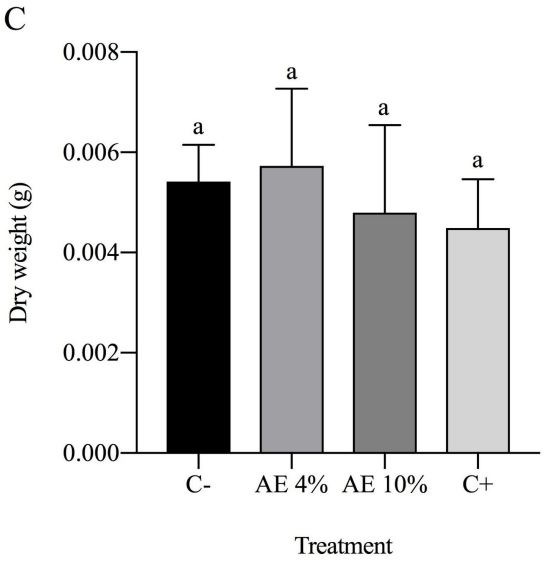


Fig. 8. Post-emergence bioassay performed in petri dishes. Effects on lettuce seedlings in the (A) shoot and (B) root length and (C) dry weight with the application of distilled water (C-), aqueous extract at 4 and 10% (AE 4% and AE 10%) and NaCl (C+). Bars represent the mean+SE. Different letters indicate a significant difference by Tukey test ($p \leq 0.05$).

3.1.4. Soil leaching bioassay

The soil leaching bioassay carried out with lettuce evaluated germination rates and plant development. In terms of seedling growth the 10% extract showed a significant reduction of shoot length, root length (Fig. 9, Fig. 10A and 10B) and dry mass (Fig. 10D). In addition, plants treated with the extracts had a dose–response decrease on chlorophyll content (Fig. 10C). Interestingly, the germination rate was higher with the 10% and 4% extract (56% and 51%, respectively) while the negative control had only 28% of seedling emergence (Fig. 11). Also, the dry weight was higher in the 4% extract compared to the control (Fig. 10D).

Fig. 9. Soil leaching bioassay. Lettuce seedlings at the end of 12 days treated with (A) distilled water - C-, (B) aqueous extract at 4% - AE 4% and (C) aqueous extract at 10% - AE 10%. Bar=1 cm.

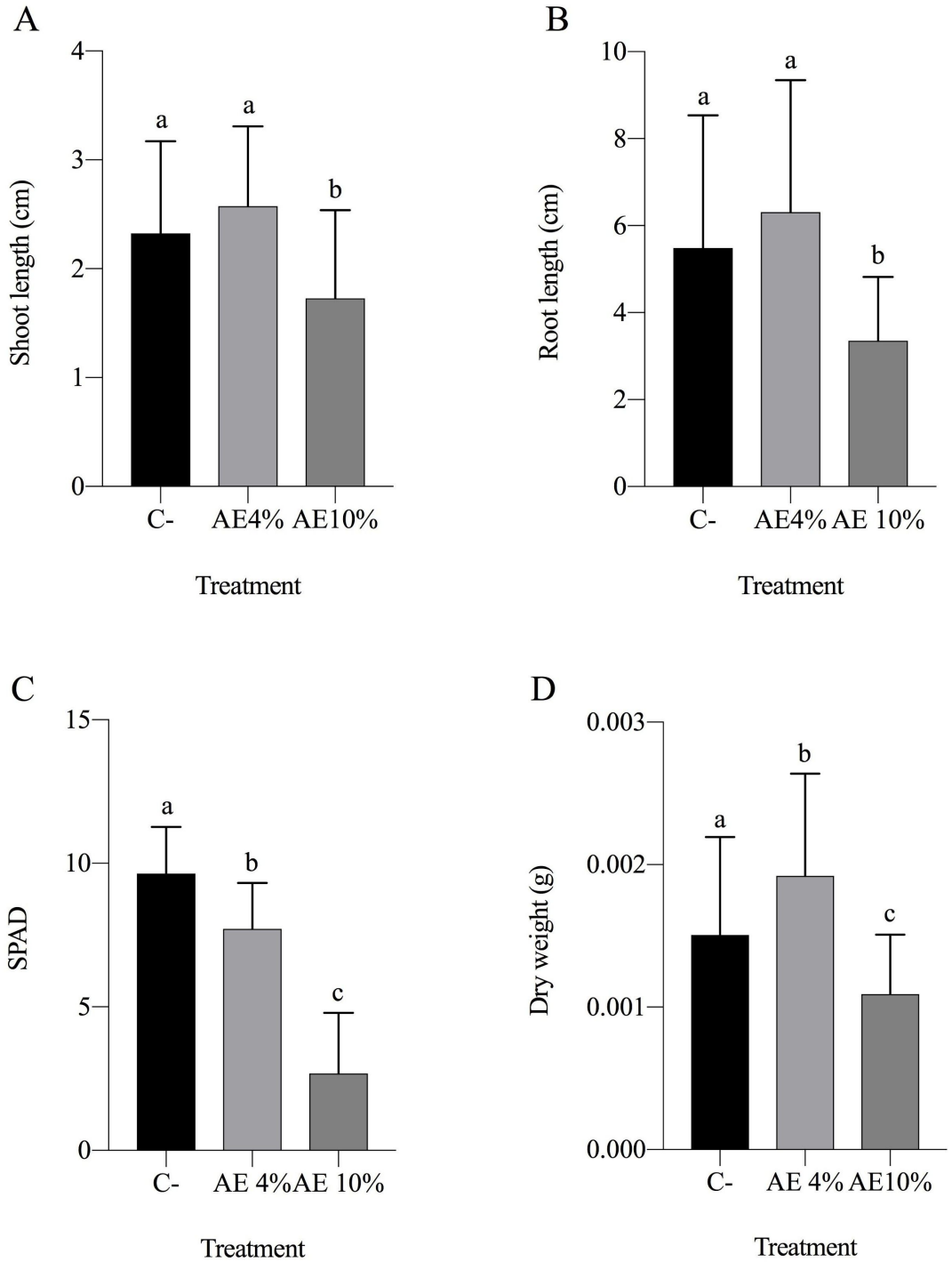


Fig. 10. Soil leaching bioassay. Seedling growth parameters of lettuce after 12 days of application of treatments (distilled water, aqueous extract at 4% and aqueous extract at 10%): (A) shoot length, (B) root length, (C) chlorophyll content and (D) dry mass of plants. Bars represent the mean+SE. Different letters indicate a significant difference by Tukey test ($p \leq 0.05$).

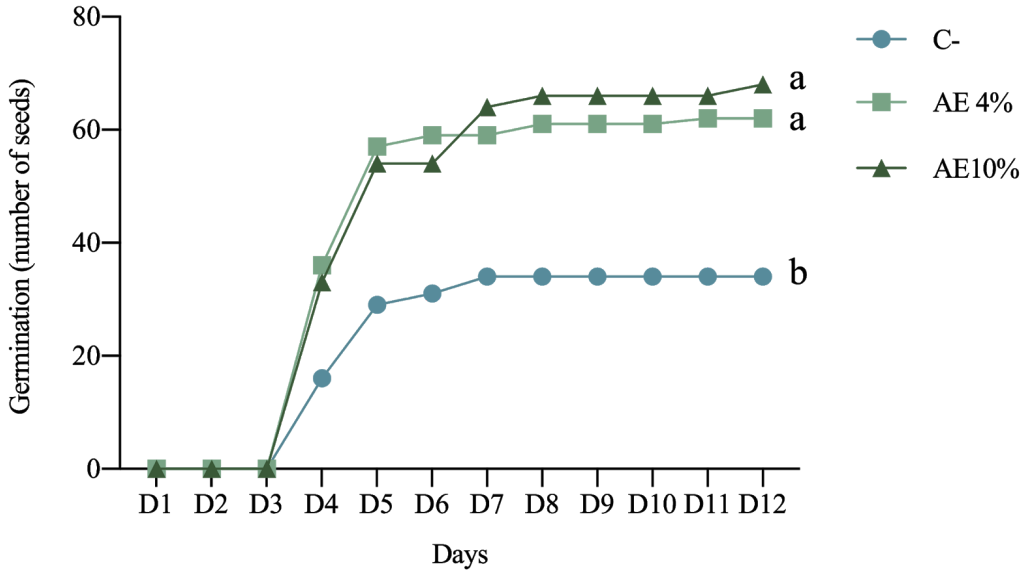


Fig. 11. Soil leaching bioassay. Germination rates of lettuce seeds in soil with the previous application of three experiment groups: distilled water (C-), aqueous extract at 4% (AE 4%) and aqueous extract at 10% (AE 10%), during 12 days of experiment.

Barnyardgrass also had a significant difference of treatments in contrast with the negative control (Fig. 12); AE 4% and AE 10% revealed a concentration-dependent effect on seedlings in terms of shoot length and dry weight (Fig. 13A and Fig. 13C).

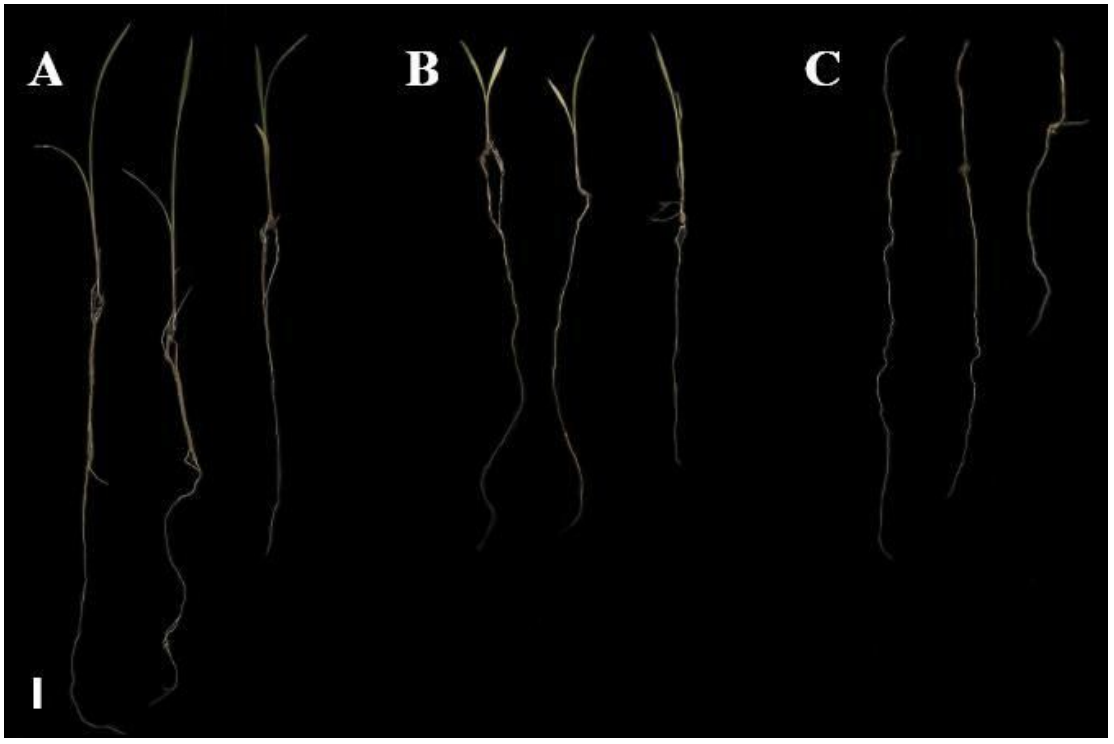


Fig. 12. Seedlings of barnyardgrass at the end of 8 days in the leaching bioassay. (A) represents negative control (plants treated with distilled water); (B) the plants with AE at 4% and (C) the plants with AE at 10%. Bar=1 cm.

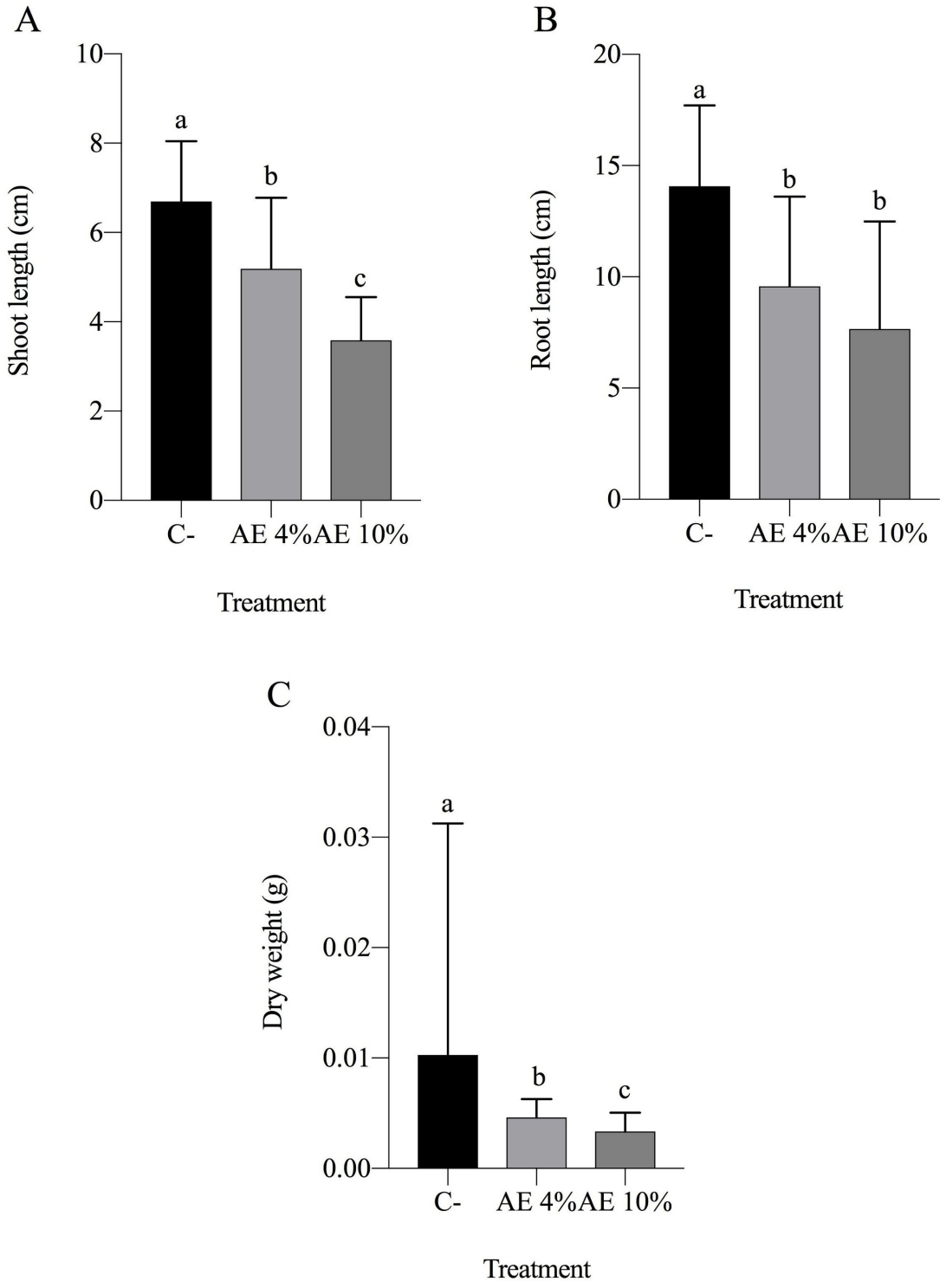


Fig. 13. Soil leaching bioassay. Seedling growth of barnyardgrass after 8 days of leaching (and 4 water applications). Evaluation consisted in measurement of (A) shoot length, (B) root length and (C) dry mass. Bars represent the mean+SE. Different letters indicate a significant difference by Tukey test ($p \leq 0.05$).

4. Discussion

4.1. Phytotoxicity assay

The pre-emergence bioassays using 4% and 10% extract of *Q. brasiliensis* against both lettuce and barnyardgrass showed a significant reduction of germination rates, root and shoot length of the emerged seedlings (Fig. 1 and Fig. 2). In *L. sativa* seeds the extracts completely inhibited germination. Extracts and QB fraction significantly delayed germination percentage, shoot and root length (Fig. 3). Considering that even in low concentrations the germination was suppressed, it appears that the extracts show phytotoxic potential that could be attributed to the saponins present in the plant. Further studies of these compounds could lead to a possible use as an effective pre-emergence bioherbicide.

Phytotoxic effect on post-emergence growth of lettuce was also observed, especially at the highest concentration of the plant extract (AE 10%) (Fig. 4). Moreover, with AE 4% treatment numerous leaves exhibited abnormalities that could explain significantly reduced chlorophyll content (Fig. 5). Although the effects on post-emergence were not as intense as those in the germination experiments, *Q. brasiliensis* extracts also have phytotoxic effects on seedling growth. In the leaching bioassay, however, there was a significant decrease in the inhibitory effect of the extracts on lettuce, particularly at 4% AE (Fig. 9). This could lead to a possible use as a post-emergence bioherbicide, alone or in combination, effective at pre and post germination with reduced residual effect.

However, the lack of significant alterations shown in the post-emergence bioassays with barnyardgrass (Fig. 6) can indicate that the extracts are not as efficient in repressing the initial growth of *E. crus-galli* as they are inhibiting its germination. It is possible that this reflects higher resistance to biotic stresses in weeds (Zimdahl, 2007). On the other hand, the leaching bioassay performed with barnyardgrass showed a statistic difference in the root and shoot length of the 10% extract treatment (Fig. 11 and Fig. 12). Perhaps for this species longer exposure to this extract residue could affect plants negatively.

Ideally, a bioherbicide residue on the soil surface should be able to undergo leaching and/or degradation, thus avoiding exposure of the subsequent crops to potentially phytotoxic compounds (Abbas et al., 2018). The leaching assay with lettuce is also considered a pre-emergence experiment, since it used seeds grown on the soil with the extracts. The higher root length and dry biomass of seedlings at 4 and 10% AE relative to the control may indicate a nutritional effect of the extracts caused, for example, by the presence of sugars in saponins

(Yendo et al., 2014). Application of the 10% extract, nonetheless, resulted in lower plant length and chlorophyll content, presumably due to a negative impact on photosynthesis (Poonpaiboonpipat et al., 2013).

Previous unpublished studies from our group pointed out the antiherbivory and antifungal effects of *Q. brasiliensis* saponins in the 1 to 2% concentration range. For the saponins to constitute a potential biopesticide, the absence of or limited phytotoxic effects at concentrations in which they are able to inhibit herbivores and pathogens is necessary. The results of the post-emergence bioassays with the 4% extract could be promising to be used as biopesticide against fungal and invertebrate herbivores with the application on seedlings, since it did not affect the plants negatively in several of the parameters.

In most cases, as has been reported for other saponins, the activity of these triterpenes depends essentially on their effects on membranes (De Costa et al., 2011). It is likely that the observed growth inhibitory effects on test plants could be also linked to cell and organelle membranes. This putative mechanism should be the focus of further research.

5. Conclusions and perspectives

Quillaja brasiliensis extracts and saponins showed strong phytotoxic activity against *Echinochloa crus-galli* and *Lactuca sativa* (with greater injury levels on the latter) and weed-suppressing abilities. Hence, further studies should be conducted to evaluate the possibility of use with adjuvants on formulations of bioherbicides. Furthermore, future work is required to better understand the mechanisms of action of the phytotoxins present in the plant. The study of new viable bioherbicides is an important step towards sustainability in agriculture and environmental damage mitigation.

6. References

Abbas, T., Zahir, Z.A., Naveed, M., Kremer, R.J., 2018. Limitations of Existing Weed Control Practices Necessitate Development of Alternative Techniques Based on Biological Approaches. Advances in Agronomy. Elsevier Inc. 147, 239-280. https://doi.org/10.1016/bs.agron.2017.10.005

Bebber, D.P., Holmes, T., Gurr, S.J., 2014. The global spread of crop pests and pathogens. Glob. Ecol. Biogeogr. 23, 1398–1407. https://doi.org/10.1111/geb.12214

Cai, X., Gu, M., 2016. Bioherbicides in Organic Horticulture. Horticulturae. 2, 3. https://doi.org/10.3390/horticulturae2020003

Caldas, E.D., 2016. Pesticide Poisoning in Brazil. Encycl. Environ. Heal. 419–427. https://doi.org/10.1016/b978-0-444-52272-6.00590-0

Carneiro F.F., Rigotto R.M., Augusto L.G.S., Friedrich K., Búrigo A.C., 2015. Dossiê ABRASCO: um alerta sobre os impactos dos agrotóxicos na saúde. Rio de Janeiro: EPSJV, São Paulo: Expressão Popular. 1-624.

Chauhan, B.S., Mahajan, G., 2014. Preface. Recent Adv. Weed Manag. 5–6. https://doi.org/10.1007/978-1-4939-1019-9

Cibulski, S.P., Silveira, F., Mourglia-Ettlin, G., Teixeira, T.F., dos Santos, H.F., Yendo, A.C., de Costa, F., Fett-Neto, A.G., Gosmann, G., Roehe, P.M., 2016. *Quillaja brasiliensis* saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice. Comp. Immunol. Microbiol. Infect. Dis. 45, 1–8. https://doi.org/10.1016/j.cimid.2016.01.004

Cordeau, S., Triolet, M., Wayman, S., Steinberg, C., Guillemin, J.P., 2016. Bioherbicides: Dead in the water? A review of the existing products for integrated weed management. Crop Prot. 87, 44–49. https://doi.org/10.1016/j.cropro.2016.04.016

Dayan, F.E., Duke, S.O., 2014. Natural Compounds as Next-Generation Herbicides. Plant Physiol. 166, 1090–1105. https://doi.org/10.1104/pp.114.239061

De Costa, F., Yendo, A.C.A., Cibulski, S.P., Fleck, J.D., Roehe, P.M., Spilki, F.R., Gosmann, G., Fett-Neto, A.G., 2014. Alternative inactivated poliovirus vaccines adjuvanted with Quillaja brasiliensis or Quil-A saponins are equally effective in inducing specific immune responses. PLoS One 9, 1–7. https://doi.org/10.1371/journal.pone.0105374

De Costa, F., Yendo, A.C.A., Fleck, J.D., Gosmann, G., Fett-Neto, A.G., 2011. Immunoadjuvant and anti-inflammatory plant saponins: characteristics and biotechnological approaches towards sustainable production. Mini Rev. Med. Chem. 11, 857-880.

De Geyter, E., Smagghe, G., Rahbe, Y., Geelen, D., 2011. Triterpene saponins of *Quillaja saponaria* show strong aphicidal and deterrent activity against the pea aphid *Acyrthosiphon pisum*. Pest management science. 68. 164-9. doi: 10.1002/ps.2235.

Duke, S.O., Dayan, F.E., Rimando, A.M., Schrader, K.K., Aliotta, G., Oliva, A., Romagni, J.G., 2002. Invited Paper: Chemicals from nature for weed management. Weed Sci. 50, 138–151. https://doi.org/10.1614/0043-1745(2002)050[0138:ipcfnf]2.0.co;2

Duke S.O., Dayan F.E., Romagni J.G., Rimando A.M., 2000. Natural products as sources of herbicides: current status and future trends. Weed Res. 40, 99–111. https://doi.org/10.1046/j.1365-3180.2000.00161.x

Faizal, A., Geelen, D., 2013. Saponins and their role in biological processes in plants. Phytochemistry Reviews. 12(4), 877–893. doi:10.1007/s11101-013-9322-4

Fleck, J.D., Kauffmann, C., Spilki, F., Lencina, C.L., Roehe, P.M., Gosmann, G., 2006. Adjuvant activity of Quillaja brasiliensis saponins on the immune responses to bovine herpesvirus type 1 in mice. Vaccine. 24, 7129–7134. https://doi.org/10.1016/j.vaccine.2006.06.059

Food and Agriculture Organization of the United Nations, 2018. FAOSTAT - Pesticides Use. http://www.fao.org/faostat/en/#data/RP/visualize (accessed 25 May 2019).

Giannakou, I.O., 2011. Efficacy of a formulated product containing *Quillaja saponaria* plant extracts for the control of root-knot nematodes. Eur J Plant Pathol. 130, 587. https://doi.org/10.1007/s10658-011-9780-8

González-Castillo, J.A., Quezada-D'Angelo, T.P., Silva-Aguayo, G.I., Moya, E.A., 2018. Effect of saponins of *Quillaja saponaria* extracts in combination with *Pseudomonas protegens* to control *Gaeumannomyces graminis* var. *tritici* in wheat. Chilean journal of agricultural research. 78(3), 378-390. https://dx.doi.org/10.4067/S0718-58392018000300378

González-Cruz, D., Martín, R.S., 2013. Molluscicidal effects of saponin-rich plant extracts on the grey field slug. Ciencia e investigación agraria. 40(2), 341-349. https://dx.doi.org/10.4067/S0718-16202013000200009

Güçlü-Üstündağ, Ö., Mazza, G., 2007. Saponins: Properties, applications and processing. Crit. Rev. Food Sci. Nutr. 47, 231–258. https://doi.org/10.1080/10408390600698197

Hakeem, K.R., Akhtar, M.S., Abdullah, S.N.A., 2016. Plant, soil and microbes: Volume 1: Implications in crop science. Plant, Soil Microbes Vol. 1 Implic. Crop Sci. 1–366. https://doi.org/10.1007/978-3-319-27455-3

Hoagland, R.E., Zablotowicz, R.M., Reddy, K.N., 1996. STUDIES OF THE PHYTOTOXICITY OF SAPONINS ON WEED AND CROP PLANTS. Sapon. Used Food Agric. 57–73.

IBAMA, 2016. Relatórios de comercialização de agrotóxicos. https://www.ibama.gov.br/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos/ (accessed 15 June 2019).

Jelassi, A., Ayeb-Zakhama, A.E., Nejma, A.B., Chaari, A., Harzallah-Skhiri, F., Jannet, H.B., 2016. Phytochemical composition and allelopathic potential of three Tunisian *Acacia* species. Industrial Crops and Products. 83, 339–345. doi:10.1016/j.indcrop.2016.01.020

Kauffmann C., Machado A.M., Fleck J.D., Provensi G., Pires V.S., et al., 2004. Constituents from leaves of *Quillaja brasiliensis*. Nat Prod Res. 18, 153–157.

Kremer, R.J., 2019. Bioherbicides and nanotechnology: Current status and future trends, Nano-Biopesticides Today and Future Perspectives. Elsevier Inc. 353-366. https://doi.org/10.1016/B978-0-12-815829-6.00015-2

Maguire, J.D., 1962. Speed of Germination—Aid In Selection And Evaluation for Seedling Emergence And Vigor 1. Crop science. 2 (2), 176-177.

Martín R., Briones, R., 1999. Industrial uses and sustainable supply of *Quillaja saponaria* (Rosaceae) saponins. Econ Bot. 53, 302–311.

Meneses, C.H.S.G., Lima, L.H.G.M., Lima, M.M.A., Pereira, W.E., Bruno, R.L.A., Vidal, M.S., 2007. Potencial Hídrico Induzido por Polietilenoglicol- 6000 na Viabilidade de Sementes de Algodão. Embrapa Agrobiologia. 19, 1-22.

Moya, E., San, R. M. Gamboa, M., & Hidalgo, G. E. A., 2010. Evaluation of a *Quillaja saponaria* saponin extract for control of powdery mildew of wheat and squash evaluación de un extracto de saponinas de *Quillaja saponaria* para el control de oídios de trigo y zapallo. Agro Sur. 38, 87-96.

Nascimento, N., Fett-neto, A.G., 2010. Plant Secondary Metabolism Engineering. 643, 1–13. https://doi.org/10.1007/978-1-60761-723-5

Oerke, E.C., 2006. Crop losses to pests. J. Agric. Sci. 144, 31–43. https://doi.org/10.1017/S0021859605005708

Pérez, A.J., Simonet, A.M., Pecio, Ł., Kowalczyk, M., Calle, J.M., Macías, F.A., Oleszec, W., Stochmal, A., 2015. Triterpenoid saponins from the aerial parts of *Trifolium argutum* Sol. and their phytotoxic evaluation. Phytochemistry Letters. 13, 165–170. doi:10.1016/j.phytol.2015.05.020

Pignati, W.A., Lima, F.A.N. de S. e, Lara, S.S. de, Correa, M.L.M., Barbosa, J.R., Leão, L.H. da C., Pignatti, M.G., 2017. Distribuição espacial do uso de agrotóxicos no Brasil: uma ferramenta para a Vigilância em Saúde. Cien. Saude Colet. 22, 3281–3293. https://doi.org/10.1590/1413-812320172210.17742017

Poonpaiboonpipat, T., Pangnakorn, U., Suvunnamek, U., Teerarak, M., Charoenying, P., and Laosinwattana, C., 2013. Phytotoxic effects of essential oil from *Cymbopogon citratus* and its physiological mechanisms on barnyardgrass (*Echinochloa crus-galli*). Ind. Crop. Prod. 41, 403–407. doi: 10.1016/j.indcrop.2012.04.057

Puig, C.G., Reigosa, M.J., Valentão, P., Andrade, P.B., Pedrol, N., 2018. Unravelling the bioherbicide potential of Eucalyptus globulus Labill: Biochemistry and effects of its aqueous extract. PLoS One. 13, 1–16. https://doi.org/10.1371/journal.pone.0192872

Reitz R., Reis, A., Klein, R.M., 1996. Flora Ilustrada Catarinense - Rosáceas. Itajaí: Herbário Barbosa Rodrigues. 113-116.

Ribeiro, R.C., de Carvalho, M.G., de Moraes, M. de L.L., Rossiello, R.O.P., de Oliveira, D.R., de Amorim, R.M.Q. and Barbieri Jr., E., 2018. Chemical Screening of *Urochloa humidicola*: Methods for Characterizing Secondary Metabolites and Allelopathic Activity on Forage Legumes. American Journal of Plant Sciences. 9, 1260-1278. https://doi.org/10.4236/ajps.2018.96093

Rice, E. L., 1984. Allelopathy. Florida: Academic Press Inc. 2, 422p.

Ross, W.C., 1974. Plant Physiology Laboratory Manual. Wadsworth Publishing Company, USA.

Sadeghloo, A., Asghari, J., Ghaderi-Far, F., 2013. Seed germination and seedling emergence of velvetleaf (*Abutilon theophrasti*) and Barnyardgrass (*Echinochloa crus-galli*). Planta Daninha. 31(2), 259-266. https://dx.doi.org/10.1590/S0100-83582013000200003

Schonbeck, M., 2011. Principles of Sustainable Weed Management in Organic Cropping Systems. 1–20.

Silveira, F., Cibulski, S.P., Varela, A.P., Marqués, J.M., Chabalgoity, A., de Costa, F., Yendo, A.C.A., Gosmann, G., Roehe, P.M., Fernández, C., Ferreira, F., 2011. *Quillaja brasiliensis* saponins are less toxic than Quil-A and have similar properties when used as an adjuvant for a viral antigen preparation. Vaccine. 29, 9177–9182. https://doi.org/10.1016/j.vaccine.2011.09.137

Stavropoulou, M.I., Angelis, A., Aligiannis, N., Kalpoutzakis, E., Mitakou, S., Duke, S.O., Fokialakis, N., 2017. Phytotoxic triterpene saponins from *Bellis longifolia* , an endemic plant of Crete. Phytochemistry. 144, 71–77. doi:10.1016/j.phytochem.2017.08.019

Stewart, C.N., 2017. Becoming weeds. Nat. Genet. 49, 654–655. https://doi.org/10.1038/ng.3851

Tirado, R., Englande, A.J., Promakasikorn, L., Novotny, V., 2008. Use of Agrochemicals in Thailand and its Consequences for the Environment. Greenpeace Res. Lab. Tech. Note 03/2008. 1–19.

Tscharntke, T., Clough, Y., Wanger, T.C., Jackson, L., Motzke, I., Perfecto, I., Vandermeer, J., Whitbread, A., 2012. Global food security, biodiversity conservation and the future of agricultural intensification. Biol. Conserv. 151, 53–59. https://doi.org/10.1016/j.biocon.2012.01.068

United Nations, 2017. World Population Prospects: The 2017 Revision. https://www.un.org/development/desa/publications/world-population-prospects-the-2017-revision.html (accessed 10 June 2019).

Weed Science Society of America, 2016. Weeds. http://wssa.net/wssa/weed/ (accessed 25 May 2019).

Wyman-Simpson, C.L., Waller, G.R., Jurzysta, M., McPherson, J.K., Young, C.C., 1991. Biological activity and chemical isolation of root saponins of six cultivars of alfalfa (*Medicago sativa* L.). Plant Soil. 135, 83-94.

Yendo, A.C.A., De Costa F., Fleck, J.D., & Gosmann, Gosmann, G., Fett-Neto, A.G., 2015. Irradiance-based treatments of *Quillaja brasiliensis* leaves (A. St.-Hil. & Tul.) Mart. as means to improve immunoadjuvant saponin yield. Industrial Crops and Products. 74. 10.1016/j.indcrop.2015.04.052.

Yendo, A.C.A., De Costa, F., Kauffman, C., Fleck, J.D., Gosmann, G., Fett-Neto, A.G., 2016. Purification of an Immunoadjuvant Saponin Fraction from Quillaja brasiliensis Leaves by Reversed-Phase Silica Gel Chromatography. Vaccine Adjuvants: Methods In Molecular Biology. 1494. 87-93. https://www.doi.org/10.1007/978-1-4939-6445-1_6

Yendo A.C.A., De Costa F., COSTA, C. T., Colling L. C., Gosmann G., FETT-NETO A. G., 2014. Biosynthesis of Plant Triterpenoid Saponins: Genes, Enzymes and their Regulation. Mini-Reviews in Organic Chemistry. 11, 292-306.

Zimdahl, R.L., 2007. Fundamentals of Weed Science, third ed. Academic Press.

Zimdahl, R.L., 2018. Fundamentals of Weed Science, fifth ed. Academic Press