

Plants extracts as germination and seedling establishment promoters in lettuce and maize

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ABSTRACT: Substances produced by plants have huge importance regulating multiple functions as germination, development promoter/ enhancer, yield increaser and activator of plant defense system against diseases and pests. Due to diversity, each plant species produces different compounds and in different concentrations. This research evaluated the role of 20 plants extracts obtained from different plant species or plant tissues on seed germination and seedlings establishment of maize and lettuce, in concentration of 5%, 10% or 20%. For this, two experiments were carried out, the first analyzing 20 plant extracts and the effects on germination percentage and root and shoot length based on the germination test for the two species. The second was carried out in a greenhouse, in a completely randomized design with three and ten repetitions for maize and lettuce, respectively, using only the three extracts with the best results from the first experiment. Seedling height and seedling dry matter were evaluated. To germination test most of extracts showed negative or no effects in both species. Only *Conyza bonariensis* at 5% and *Richardia brasiliensis* at 5% and 20% had positive effects on early seedling growth, increasing the seedlings length for maize seedlings. To seedlings establishment, *Conyza bonariensis, Leucaena leucocephala* and *Richardia brasiliensis* extracts did not show statistical difference to shoots height but increased the dry mass of shoots and roots. The results demonstrated that these plants extract have potential as growth promoters and can be a good option to a better seedling growth and establishment, collaborating to a more effective agriculture. **Key words**: biostimulants, *Lactuca sativa* L., Seedlings growth, *Zea mays* L.

Extratos vegetais como promotores de germinação e estabelecimento de plântulas de alface e milho

RESUMO: Substâncias produzidas por plantas têm grande importância na regulação de múltiplas funções, como germinação, promoção de crescimento, aumento na produção (frutos, sementes, biomassa, etc.) e ativação de mecanismos de defesa da planta contra ataques de pragas e doenças. Plantas são amplamente diversas, cada espécie produz diferentes compostos em diferentes concentrações. O objetivo deste trabalho foi avaliar o efeito de 20 extratos de plantas obtidos de diferentes espécies vegetais sobre a germinação e estabelecimento de plântulas de milho e alface, nas concentrações de 5%, 10% ou 20%. Foram realizados dois experimentos, o primeiro afim de avaliar os efeitos dos 20 extratos sobre a germinação e sobre o comprimento radicular e de parte aérea após germinação. Para o segundo experimento, conduzido em delineamento inteiramente casualizado com três e dez repetições para milho e alface, respectivamente, apenas os três extratos mais expressivos foram selecionados. A altura e massa de matéria seca das plântulas foram avaliados. Para o primeiro experimento a maioria dos extratos demonstraram efeito negativo para a germinação en ambas as espécies vegetais. Apenas *Conyza bonariensis* a 5% e *Richardia brasiliensis* a 5% e 20% tiveram efeitos positivos, aumentando o comprimento de plântulas de milho pós germinadas. Para o estabelecimento de plântulas, extratos de *Conyza bonariensis, Leucaena leucocephala e Richardia brasiliensis* não apresentaram diferença estatística para altura de parte aérea, mas potencial como promotores de crescimento, podendo assim ser uma boa opção para o crescimento e estabelecimento de plântulas, colaborando para uma agricultura mais limpa e efetiva.

Palavras-chave: bioestimulantes, Lactuca sativa L., Crescimento de plântulas, Zea mays L.

INTRODUCTION

With the increasing in the world population, the raise in the productivity of food to supply the demand around the world is required, that fact put the modern agriculture to face challenges as limited amount of area enable to agriculture and yield losses due to pests and diseases (FAO, 2018). Furthermore, the world agriculture is changing aiming for less damage to environment and efficient use of available resources, with that, the seek for environmental-friendly and sustainable technologies is a tendency. Thus, substances extracted from some plants can have an important role to benefit other plants development (ROUPHAEL & COLLA, 2020) and response to stress (FAROOQ et al., 2017).

A biostimulant or plant growth regulator may be any substance or mixture of substances which improves the condition of crops without causing adverse side effects (YAKHIN et al., 2017). The main active substances used as plant growth regulator are humic and fulvic acids, protein hydrolysates,

Received 08.09.23 Approved 04.04.24 Returned by the author 06.14.24 CR-2023-0436.R2 Editors: Leandro Souza da Silva[®] Jean Possenti[®] compound containing nitrogen, seaweed extracts (BOUKHARI et al., 2020), beneficial fungi, bacteria (DROBEK et al., 2019) and plant secondary metabolites (DU JARDIN, 2015).

Plant growth regulators can collaborate throughout the crop life cycle, by stimulating root elongation, increasing plant tolerance to abiotic stresses and the recovery from it, enhancing crop standard and quality attributes of production (CHEN et al., 2018), also plant growth regulators can improve the efficiency of plant's metabolism to induce yield increases and enhanced crop quality (SHAHZAD et al., 2018); better nutrient assimilation, translocation and use; enhancing quality attributes of produce and rendering water use efficient (NAGY et al., 2019).

Recent studies demonstrated the potential of plant crude extracts as plant growth regulators. CULVER et al. (2012) reported that application of crude extract of *Moringa oleifera* leaves on tomato leaves two weeks after germinating could increase growth and yield, dry roots weight, and height of tomatoes plants. ABDALLA (2013) reported that application of 2% leaf extract and 3% branch extract of *Moringa oleifera* with the frequency of two times (7 and 14 days after planting) in a planting season, significantly increased height, fresh and dry weight of *Eruca vesicaria* subsp. *sativa*. ERTANI et al. (2015) found that 50 mg.L⁻¹ of grapefruit peel extract sprayed at two and four weeks after planting could increase biomass and dry weight of chili pepper.

Maize (*Zea mays*) is one of the most important world's commodities, being the second highest crop in production around the world and a staple source of food and energy to many countries (CONAB, 2019). Lettuce (*Lactuca sativa*) is the most consumed leafy vegetable in Brazil due to its low cost and easy acquisition in the whole year, furthermore it is a socially and economically important crop to small producers and in Brazil (CONAB, 2017) and in other countries (ANSARI et al., 2020).

Given the necessity for new products capable of enhance crops development and lack of information of plant components as plant growth regulator, this research had the objective to evaluate the potential of different plants and plant tissues extracts to promote germination and improve establishment of maize and lettuce seedlings.

MATERIALS AND METHODS

The experiment was divided in two, the first one to evaluate the seed germination and early seedling growth by the length evaluation of maize and lettuce submitted to 20 extracts from different species and tissues as seeds, leaves and fruit peel in concentrations of 5%, 10% and 20% of crude extract in established volume of water. The extracts with best results were selected to the second experiment, which had the objective of evaluate the establishment of seedlings of maize and lettuce treated with these extracts. For both experiments, the control treatment was done just with water.

The extracts were selected according with the potential of each species in collaborate with early growth of the crop evaluated, the plant component as source of extract was selected based on the possibility of each vegetal tissue produce different quantities of metabolites. Developing plant tissues can present high quantities of compounds and a more efficient extraction, agroindustry residues are interesting sources due to the possibility of creating value to it, and giving it a new destiny, so that decreasing industries wastes.

The following plant extracts were selected from leaves: Leucaena leucocephala (Lam.), Moringa olerífera (Lam.), Rosmarinus officinalis L., Ipomoea purpurea L. Roth, Talinum paniculatum (Jacq.) Gaertn, Euphorbia hirta L., Amaranthus viridis L., Ricinus communis L., Mentha spicata L., Laurus nobilis L., Cyperus rotundus L., Cymbopogon citratus (D.C.) Stapf, Peumus boldus Molina, Richardia brasiliensis Gomes, Conyza bonariensis (L.) Cronquist and Oxalis latifolia Kunth. The seed extracts were from Citrus sinensis (L.) Osbeck, Persea americana Mill. and Ricinus communis L. and those from peel were of Citrus sinensis (L.) and Persea americana Mill. The leaves used for the extraction were totally expanded, the seeds and peel were collected from ripe fruits. The plants were maintained on field.

The extracts were obtained by maceration using crucible and pistil and immersion in hydroalcoholic solution in 1:3 proportion (weigh: weighh), with a final weight of 400g (100g of water and 300g of alcohol). It was used 20g of each washed out vegetal material (corresponding to 5% of solution total weight) as described in the previous paragraph. The material was collected and used fresh, following MARINHO et al. (2018) adapted methods. The material was macerated, allocated in hydroalcoholic solution, and stored seven days in dark room, after that the solution was filtered and submitted to rotary evaporator at 70 rpm and 60 °C of temperature at low pressure for 35 minutes, to remove all the alcohol from the solution. The extract obtained was stored in a freezer at -20 °C, to use they were unfrozen at room

temperature and from the crude extract the formulation of concentrations were done with tap water.

The first experiment was conducted in a completely randomized design with a 21x3 factorial scheme (20 extracts and control x three concentrations) with four repetitions for each combination. A control treatment using just water was added.

To evaluate the germination and length of seedlings, it was used the germination test according to Seeds Analyses Rules (BRASIL, 2009), to both evaluated species, four repetitions of 50 previously untreated seeds for each treatment was used.

To lettuce seeds were used gerbox, using two blotting papers moistened with extract or water (control) in a proportion of 2.5x the dry paper mass. To maize, rolls made with three germination paper sheets were used as substrate, moistened with extract or water (control) in a proportion of 2.5x the dry paper mass. The boxes and rolls were covered with plastic bags and allocated in germination chambers at 25 °C. For first germination count, evaluations were done in the fourth day since installation of experiment and for germination in the seventh day for both species (BRASIL, 2009). At the seventh day since the installation of experiment, ten seedlings were randomly selected for length evaluation. To maize, the main root and shoot were measured, obtaining the total length, while to lettuce only the total length was measured.

For the second experiment, after germination test, the three best extracts were selected to be tested in the seedlings establishment. This experiment was conducted in a completely randomized design with a 3x3+1 factorial scheme (3 extracts x 3 concentrations + control). The control (just water) was considered as isolated treatment because it did not present levels.

Maize seeds were sown in plastic tray, after seven days they were transplanted in five litters pots filled with soil and vermiculite, each repetition was formed by one pot with two seedlings, it was used three repetitions, summing a total of six plants per treatment. As for the lettuce, the seeds were sown in plastic trays, each cell of the plastic tray was considered a repetition, a total of ten cells were used per treatment.

The seedlings were maintained in greenhouse, the concentrations of extracts applied were the same than the first test (5%, 10% and 20%). Fourteen mL of solution per repetition were sprayed using a hand sprayer in the seedlings leaves seven days after experiment installation, and sequential sprays were done in an interval of seven days until a total of 28 days to maize and 14 days to lettuce (summing a total of four applications to maize and two to lettuce).

After last application, the seedling height and dry mass were used as parameters to evaluate the seedling establishment. The seedling height was evaluated considering the plant base until de up to the last stretched leaf. For seedling dry mass, an oven with forced air circulation at 65°C was used until the mass stabilized.

In the statistical analyses, for the first experiment an analysis of variance (ANOVA) was performed and, in the case of significant differences ($P \le 0.05$), the Tukey test was applied, and the results were grouped in an infogram, divided in positive effect (when higher results compared to control), indifferent (no significant difference from control) and negative effect (when lesser results compared to control). For the second experiment an ANOVA and Tukey test were done, disregarding the isolate effect of control, all statistics analyses were made using statistical software R.

RESULTS

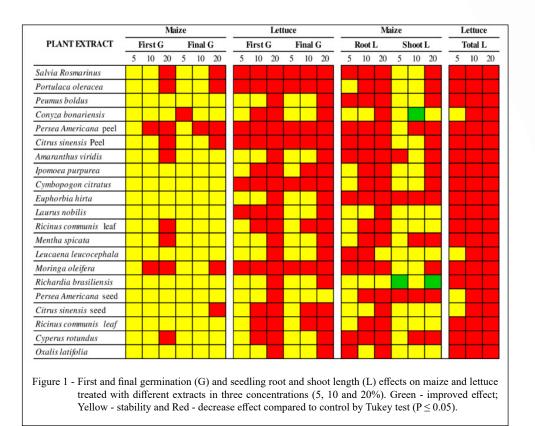
To easier and better comprehension, the results are showed as a figure comparing the extracts in each concentration with the control treatment, those with green colors means improvement in the evaluated parameter, while yellow means no difference (stability) and red decrease in it (Figure 1), this separation was made based in the Tukey test analyses, with a significant difference of 5%.

To first and final germination of maize none of the extracts showed improvements, many of them showed results equal to control treatment in all concentrations. A tendency can be seen in some extracts, that is the increase in concentration resulted in the decreasing of germination (Figure 1).

To lettuce seeds there were no increases in germination and a downward trend was observed in germination with the increasing of the extract concentration(Figure 1). The same as observed formaize.

None of the extracts showed improvements to root length, instead, all of them decreased it in at least one concentration (except to *Richardia brasiliensis*) and the others maintained equal to control measures (Figure 1).

Notable results were obtained to shoot length for maize. Extract of *Conyza bonariensis* increased it at 10% concentration, while *Richardia brasiliensis* increased it at 5% and 20%. These results showed that each extract behave differently, probably due to quantity and diversity of compounds, its concentrations even with small adjustments can have a totally different interaction. Most of the extracts



had neutral effect to shoots for maize, the highest concentration in many extracts reduced the shoot length, like germination and root length, but compared to these parameters, no tendency between increasing concentration and decreasing length was observed (Figure 1). Extract of *Leucaena leuocephala* showed neutral effect in most situations, for both maize seeds and lettuce (Figure 1).

Regarding the lettuce seedlings length, a drastic reduction was observed in all concentrations and extracts applied, as the germination test result. No positive effect was observed. The reduction in the seedlings length is directly related to the low germination percentage, so that, even if germinated, the seedling has its early growth affected (Figure 1). This result in comparison to obtained in maize seeds has a huge difference, observing this fact is important to question why this difference is happening and to understand how different seeds behave to extracts applications.

To evaluate the establishment of seedlings only three extracts were selected. *Conyza bonariensis* and *Richardia brasiliensis* were chosen due to the positive effects observed for maize seeds (Figure 1). *Leucaena leucoceplaha* was maintained because it was the extract that presented the greatest amount of neutral effect, both for corn and lettuce (Figure 1). Therefore, we proceeded with the evaluation of seedling establishment, aiming to identified possible effects that were not evident in the previous evaluation. It was decided to maintain the evaluation of lettuce seeds to enable the effect of the extracts on seeds with different morphological characteristics, which could impact the observed response.

Regarding the germination of maize, the only statistical difference is for Conyza bonariensis at 5%, where a decrease of 10 and 7.5 percentage points is observed in relation to extracts of R. brasiliensis and L. leucocephala, respectively (Table 1). In root length different behavior is observed between the extracts. Convza bonariensis at a concentration of 5% increased root length by 95% compared to L. leucocephala and decreased at higher concentrations. It was observed the opposite for Leucaena leucocephala, lower concentration showed smaller roots and higher concentrations bigger roots. L. leucocephala extract at a concentration of 20% increased root length by 125% when compared to the C. bonariensis extract at the same concentration. To Richardia brasiliensis no pattern is observed, each concentration acted differently (Table 1).

5

Germination (%)				Root length (cm)			
Extract	5%	10%	20%	5%	10%	20%	
C. bonariensis	88.0 Bb	96.0 Aa	96.0 Aa	15.44 Aa	11.41 Ba	5.58 Cb	
L. leucocephala	95.5 Aab	96.5 Aa	98.0 Aa	7.90 Bc	8.21 Bb	12.57Aa	
R. brasiliensis	98.0 Aa	96.5 Aa	97.5 Aa	12.08 Ab	10.53 Ba	11.85Aa	
Control		97.5			13.74		
CV (%)		14.87			12.54		
Shoot length (cm)				Plant height (cm)			
Extract	5%	10%	20%	5%	10%	20%	
C. bonariensis	8.0 Bb	9.55 Aa	8.35 Bb	106.87Aa	100.0 Aa	101.92Aa	
L. leucocephala	7.56 Ac	7.71 Ac	7.77 Ac	106.71Aa	104.75Aa	102.7 Aa	
R. brasiliensis	9.02 Aa	8.44 Ab	9.50 Aa	98.88 Aa	84.63 Aa	103.71Aa	
Control		6.22			103.3		
CV (%)		15.21			15.32		

Table 1 - Germination, root and shoot length and plant height of maize treated with Conyza banariensis, Leucaena leucocephala and Richardia brasiliensis extracts in three concentrations (5, 10 and 20%).

Averages followed by same uppercase letters in lines and lowercases letters in columns means no statistic difference between them by Tukey test at 5% of probability. CV (%): Coefficient of variation.

To shoot length, most of extracts at lowest concentration had stability, the more expressive results are shown to Conyza bonariensis at 10% and to Richardia brasiliensis at 5% and 20% (Table 1). Regarding maize establishment (plant height) no statistic difference was observed between treatments (Table 1).

Lettuce seed germination was higher in treatments with L. eucocephala and R. brasiliensis extract when compared to C. bonariensis extract in the concentrations of 10% and 20% (Table 2). When used at a concentration of 5%, the extracts of L. eucocephala and R. brasiliensis were similar to the control. The percentage of germination was reduced when the extracts were applied at a concentration of 20%, mainly that of C. bonariensis, which reduced between 48.5 and 50.5 percentage points in relation to the

other extracts and 78.5 when compared to the control (Table 2). About the lettuce, the seedling demonstrated a decrease in length according to concentration increases, none of the extracts showed higher results than the control, only stability and negative results were obtained (Figure 1). Plant stablishment test increment was observed to Leucaena leucocephala at 5% and Richardia brasiliensis at 10%, but this increment had no statistical significance (Table 3).

The shoot dry matter was obtained as a unique sample, by weighing all plants together. All extracts in all concentrations showed increments to dry matter of maize shoots (Figure 2A), to Conyza bonariensis the best results were obtained in 5% and 10% getting the double of weight compared to plants with no treatment. To Richardia brasiliensis the best

Table 2 - Germination of lettuce treated with Conyza banariensis, Leucaena leucocephala and Richardia brasiliensis extracts in three concentrations (5, 10 and 20%).

Germination (%)			
Extract	5%	10%	20%
C. bonariensis	91.0 Aa	85.5 Ab	19.0 Bb
L. leucocephala	96.5 Aa	90.5 Aa	67.5 Ba
R. brasiliensis	94.5 Aa	91.5 Aa	69.5 Ba
Control		97.5	
CV (%)		8.94	

Averages followed by same uppercase letters in lines and lowercases letters in columns means no statistic difference between them by Tukey test at 5% of probability. CV (%): Coefficient of variation.

Seedling height (cn	n)		Seedling length (cm)			
(Seedling establishment)				(Early seedling growth)		
Extract	5%	10%	20%	5%	10%	20%
C. bonariensis	6.7Ab	6.69Aab	6.03Ab	4.42Aa	3.99Ba	2.00Cb
L. leucocephala	8.3Aa	6.04Bb	6.83ABa	4.46Aa	3.53Bab	2.76Ca
R. brasiliensis	5.75 Cc	7.73Aa	6.58Bab	3.83Ab	3.05Abb	2.64Ba
Control		7.5			5.80	
CV (%)		13.82			12.32	

Table 3 - Seedling length and seedling height of lettuce treated with *Conyza banariensis*, *Leucaena leucocephala* and *Richardia brasiliensis* extracts in three concentrations (5, 10 and 20%).

Averages followed by same uppercase letters in lines and lowercases letters in columns means no statistic difference between them by Tukey test at 5% of probability. CV (%): Coefficient of variation.

results was obtained in 5% of concentration, and to *Leucaena leucocephala* the opposite was observed, getting the best result in the highest concentration.

Similar results were observed to roots dry matter in relation to concentration. To *Conyza bonariensis* 10% was the best concentration, increasing the roots matter 3x more than plants without treatment, while to *Richardia brasiliensis* was 5% and to *Leucaena leucocephala* 20% (Figure 2B).

To evaluate the lettuce dry matter the whole plant was weighted, *Conyza bonariensis* and *Leucaena leucocephala* showed similar results to same concentrations, getting best results at 20% of concentration, to *Richardia brasiliensis* the best was obtained at 10%, being the highest dry mass of all treatments (Figure 3). The results reinforce the idea that small adjustments in concentration cause big effects to plant growth.

DISCUSSION

For many extracts, it was observed that an increase in extracts concentration resulted in the decreasing of germination, probably due to allelopathic compounds (LI et al., 2021; TEIXEIRA et al., 2018) or effects of osmotic potential, as shown by SERT & KOKUBO (2017) when high concentrations of sucrose in water were applied in soybean seeds. In other hand the extracts can help against some stresses and contribute to a better plant performance (DESOKY et al., 2019; EL-MAGEED et al., 2017), as shown by FIAZ et al. (2018) when *Moringa oleirifera* extract was applied as seed priming in *Celosia cristata* L to evaluate its effectiveness to germination under different salinity levels.

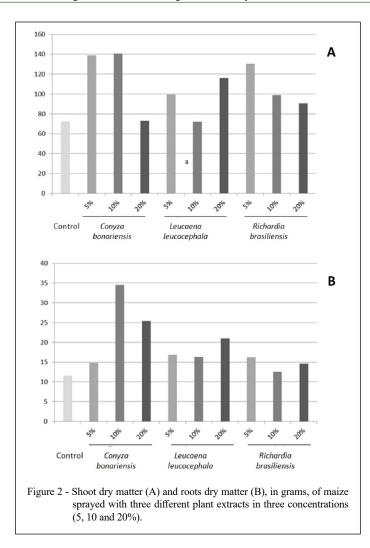
Even with no increasing, the results obtained to final and first germination of maize collaborated

demonstrating that some of the evaluated extracts (those with neutral effects) have potential as selective bioherbicides to maize, controlling weeds without damaging the main crop, as shown in experiment using *Leucaena leucocephala* in maize development (PRATES et al., 2000) and in studies evaluating the effect of *Eucalyptus camaldulensis* extract, where the application showed satisfactory results in suppressing weeds and enhancing yield of maize (KHAN et al., 2020). Even with promissory results, more studies with the evaluated extracts must be done to conclude its real potential, benefits and limitation to maize.

The huge difference between maize and lettuce germination, even that the volume applied was the same can be due to seeds size. Lettuce seeds have bigger contact area related to seed volume, what allows a faster reaction with substances and damage to embryo, while the maize has a smaller contact area and the presence of huge endosperm, that can act as a barrier protecting it (COSTA et al., 2004).

Therefore, the shoots of maize have better response to extracts application compared to roots, these results are similar to those obtained by TANASE et al. (2018), in which spruce bark extracts were applied in *Ocimum basililicum* seedlings. The application positively affected both root and shoot growth with a better expression in this second, increased the biomass accumulation, photo-assimilating pigment synthesis, collaborated to an intensification of metabolic processes and cell division, higher mitotic index and good development of vascular bundles, probably due to the substances uptake or the affinity of these substances with the applied area.

To evaluate the establishment of seedlings three extracts were selected. *Conyza bonariensis* was selected due to its increasing in shoot size of



maize seedlings in one concentration and stability to lettuce in the lower one, to *Richardia brasiliensis* this parameter was used too, due to two concentrations showing increases to maize shoot length. *Leucaena leucocephala* did not show increment in plant length but demonstrated a consistence to germination in both seedlings tested and stability to lettuce in the lower germination (what did not happen in most extracts).

It was observed difference on establishment responses between the different extracts and concentrations for maize seeds. Other studies showed the different response of plants to extracts, PHIRI (2010) using *moringa oleirifera* leaves extract obtained increasing to radical length of maize but reduced to rice, wheat and sorghum.

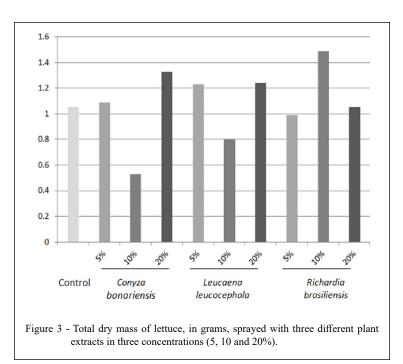
The increasing in height demonstrated in *Conyza bonariensis* and *Richardia brasiliensis* could be due to hormones and other substances present in the extracts, like auxins (ROUPHAEL et al., 2021), collaborating to the seedling growth.

Extracts seems to behave differently according to the area where they are applied, being able to promote enhances to the whole plant, as observed with the application of *aloe vera* leaf extract in aspen trees (*Populus* spp.) (EL SHERIF, 2017), or enhance a specific section of the plant, as MUTLU-DURAK & KUTMAN (2021) observed with the application of willow (*Salix babylonica*) bark and leaf extract in maize seedlings, both enhanced growth and stablishment, but better responses were observed in numbers to shoots compared to roots in development.

All extracts in all concentrations showed increments to dry matter of maize shoots. Even that no significant increment was observed in plant height, the shoot dry matter showed good response to extract application, that can be due to an efficiency gain in photosynthetic conversion (TANASE et al., 2018).

Similar results were observed to roots dry matter. The increasing in the roots matter is important to a better exploration of water and nutrients and to

Silva et al.



the plant gets a better structure and fixation in soil, collaborating directly to its better development and establishment (CAMPOBENEDETTO et al., 2021).

Studies of lettuce development when treated with *Scenedesmus quadricauda* extract demonstrated biostimulant effects to seedlings roots and shoots height, dry weight, and number of leaves (PUGLISI et al., 2020), the results obtained in these different experiments make clear the importance of the extraction method, concentrations, plant species, plant phenology and area of application as a determinant if the extract will contribute or disturb the plant development.

Studies made by TANASE et al. (2018) show that plant extracts can increase different components of plants, above-ground as stem and leaves have better responses to it, according to substances present in the extracts, but good responses can be obtained since the substance is known, as the knowledge of its act in each component of the plant.

This experiment collaborated to bring new questions about plants extracts and its capacity as bioregulators or bioherbicides, to better comprehension different concentrations need to be tested, as an in deep study of each plant extract is needed to identify, isolate and quantify the components of them, so that efficient methods to work with each one can be obtained.

CONCLUSION

To germination test most of extracts showed negative or no effects in both species. Only *Conyza bonariensis and Richardia brasiliensis* had positive effects on early seedling growth, increasing the seedlings length for maize seedlings. For plant establishment tests, positive results were obtained on shoot length of maize and on dry matter of both species at least in one concentration to Conyza bonariensis, Leucaena leucocephala and Richardia brasiliensis extracts.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

LES and GZM designed the experiment and carrier on the evaluations on laboratory and greenhouse. ASG reviewed the manuscript. VAF designed the experiment and supported with planning and adjustment during all the experiment process. LES,

ASG and VAF prepared the article for submission. All authors critically reviewed the manuscript and approved the final version.

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10