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Article

PHYTOTOXIC POTENTIAL OF *Ipomoea batatas* EXTRACT, DETECTED THROUGH A NEW TYPE OF SANDWICH MICROBIOASSAY ON THREE SPECIES OF WEEDS

*Potencial Fitotóxico de Extratos de **Ipomoea batatas**, Detectado Por Meio de um Novo Microbioensaio do Tipo Sanduíche, em Três Espécies de Plantas Daninhas*

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ABSTRACT - Nowadays, it is very important for researchers to find alternatives that enable the development of a profitable agriculture and a clean environment, therefore, the strategy evaluated in the present study is geared towards the use of the allelopathic effect of many plants. The main objective was to assess the efficiency of a compound derived from extracts of sweet potato (*Ipomoea batatas* on three weed species under controlled conditions. Different concentrations (1, 5, 10% w/v) were evaluated through a sandwich-type microbioassay in comparison with the traditional *Petri* dished culture technique. Both tests demonstrated the phytotoxic activity of aqueous extracts of *I. batatas*, which caused inhibition of germination of *A. hybridus, P. oleracea and B. campestri*. It was observed that use of the microbioassay allowed the optimization of resources used for analysis, required much less amounts of extracts and facilitated the analysis of a larger number of samples per unit of time. This offers a new economic and efficient alternative to quickly assess the phytotoxic effect of many donors' species before field tests.

Keywords: allelopathy, sweet potato, residues, *A. hybridus, P. oleracea, B. campestri*.

RESUMO - Atualmente é muito importante pesquisar e encontrar alternativas que permitam desenvolver uma agricultura rentável e um ambiente limpo, por isso, a estratégia adotada neste estudo foi a utilização do efeito alelopático de maior número de plantas. O objetivo principal foi avaliar a eficácia de um formulado a partir de extratos de batata-doce (Ipomoea batatas), com três espécies de plantas daninhas, sob condições de estufa. Diferentes concentrações do formulado foram avaliadas (1, 5 e 10% p/v) por meio de um novo microbioensaio do tipo sanduíche, em comparação com a tradicional cultura em placas de Petri. Ambos os estudos demonstraram a atividade fitotóxica de extratos aquosos de I. batatas para inibir a germinação de A. hybridus, P. oleracea e B. campestri. Foi observado que o uso de microbioensaio permitiu a otimização de recursos para análise, requereu menos extrato e facilitou a análise de maior número de amostras por unidade de tempo. Esse resultado oferece uma nova alternativa econômica e eficiente para testar rapidamente o efeito fitotóxico de maior número de espécies de doadores antes do teste de campo.

Palavras-chave: alelopatia, batata-doce, resíduos, *A. hybridus, P. oleracea, B.campestri*.

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INTRODUCTION

Weeds can cause significant losses in major crops, estimated to be between 40-45% in leafy green vegetables, 35-40% in chili, 30-35% in okra, 25-35% in tomato, 25-30% in eggplant, 15-25% in cauliflower, 15-20% in cowpea and cucurbits (Singh et al., 2014).

The use of allelopathic residues derived from *Sphagneticola trilobata* as a crop management tool may be one of the most practical uses of allelopathy for the integrated weed management in agriculturally important crops (Hernández-Aro et al., 2016). These natural interactions produce multiple effects, ranging from inhibition or stimulation of the growth processes of neighboring plants to inhibition of germination. It has been observed that aqueous extracts of the residues of those plant species may have a negative allelopathic potential on different cultures and this is due to the allelochemical content of the extracts, the application time and the concentration used. Therefore, their natural derivatives form a very important part of the plant defense systems, besides offering benefits, such as biodegradation (Blanco-Valdes, 2006, 2016).

It has been reported in the specialized literature that the sweet potato (*Ipomoea batatas*) exhibits phytotoxic effects in plants and microorganisms. Torres et al. (2003) observed stimulating effects on different crops, including gourd (*Cucurbita* sp.), melon (*Cucumis melo*), corn (*Zea mays*), sorghum (*Sorghum bicolor*), cucumber (*Cucumis sativus*) and radish (*Raphanus sativus*), while inhibition was observed in bean (*Phaseolus vulgaris*) and in weeds such as wild poinsettia (*Euphorbia hetearophylla*) spreading amaranth (*Amaranthus crassipes*), purslane (*Portulaca oleracea*) and jungle rice (*Echinochloa colona*).

Generally, the procedure found to study the effect on seeds and host plants, using extracts and chemical substances, is based on Petri dishes, as this has been used as evidence of the presence of allelochemicals in donor plants (Xuan et al., 2004; John et al., 2006; Oliveros-Bastida et al., 2011; Xuan et al., 2016).

Other techniques have been recently described by Fuji (2003) and Miyaura (2012), who mentioned the sandwich method, given its similarity to a sandwich, in which samples of residues or extracts are placed between two agar layers, on a culture holder or a six-well plastic culture plate (10 mL each), (Fuji et al., 2004; Itani et al., 2013) cited by Hernández-Oro et al. (2015a).

Preliminary reports have demonstrated the allelopathic potential of *I. batatas* as a donor plant on some host species such as *A. palmeri*, using a sandwich microbioassay, developed on an ELISA plate. The optimized test requires a minimal level of resources per analysis, less amounts of donor extract, performing more analyses per unit of time (Hernández Aro et al., 2015a). When implementing a bioassay, it is necessary to carry out its standardization, which consists in establishing the sensitivity of the species and the reproducibility of the experiment in relation to a xenobiotic compound used as a reference. The standardization enables comparison of data coming from different laboratories, and then through the calibration process, a quality control is established to achieve higher precision results in a particular bioassay (Oliveros-Bastida et al., 2011). Given the issues at stake, the evaluation of *Ipomoea batatas* extracts through the conventional Petri dishes bioassay technique and the new method for bioassay, the sandwich technique, will allow one to outline the efficiency of both techniques in detecting the phytotoxicity and allelopathic capacity of host weed species.

The objective of the present work was to determine the phytotoxic potential of the *Ipomoea batatas* extract through a new microbioassay, the sandwich method, compared with the traditional Petri dish culture technique, on three recipient species.

MATERIALS AND METHODS

The study was conducted during the months of May-August 2015. The species: *Amaranthus hybridus, Brassica campestri* and *Portulaca oleracea*, were used as recipients during the tests, and compared at different concentrations of *I. batatas* extracts.

Plant material and collection of the plant extract

The processing of the plant material was performed according to the methodology reported by Narwal (1996), quoted by John et al. (2006) and Espinosa et al. (2012). Once the stems, leaves and flowers were collected, they were rinsed with abundant water, then dried in a bacteriological incubator (Riossa digital) for 48-72 hours at a temperature of 45 °C. The dried residues of *I. batatas* were ground twice into particles with approximately 1mm in diameter, using a hand grain mill (Estrella company), made in Mexico. The ground plant tissue was stored in polyethylene bags, under dark conditions and low humidity until its use.

The extract of *I. batatas* was obtained by soaking mashed residues (1:10 w/v) in 60 mL of distilled water and kept under the agitation rate of 300 rpm for 24 hours, under dark conditions at room temperature (22 ± 2 °C). The mixture was filtered using a Whatman Grade 1 filter paper, and then it was obtained a stock solution, from which the rest of the extract treatments were prepared by dilution at a concentration of 1 and 5% w/v. The extracts were kept under refrigeration at 4 °C until needed.

Sterilization of the material and disinfection of seeds

Soil, water and Petri dishes (∅ 150 mm, h = 25 mm) containing a Whatman Grade 1 filter paper were autoclaved (RayPa, Spain) at 120 °C and a pressure of 1.5 atm for 30 minutes. The material was previously dried (45 °C, 24 hours) in a bacteriological incubator and then used.

The seeds of *A. hybridus*, *B. campestri* and *P. oleracea* were donated by the Weed Science Department of the Chapingo Autonomous University (UACh), Mexico State, in Mexico. These were collected in November 2013 *(B. campestri* and *P. oleracea*) and in October 2014 (*A. hybridus*), at the Xaltenpan Experimental Field of the UACh. They were previously disinfected via immersion in 3% sodium hypochlorite for 5 minutes. Subsequently, they were washed three times in sterile distilled water for 10 minutes, and then were ready for use.

Detection of *I. batatas* phytotoxicity through bioassays

The treatments consisted of three different concentrations of aqueous extracts of *I. batatas*: 1, 5, 10% w/v and a control with sterile distilled water.

Each experimental unit was set up with a Petri dish (control) against a 24-well section of a removable reaction plate (Nunc, USA), where 24 seeds of the host species were placed: *A. hybridus*, *B. campestri* and *P. oleracea* (a total of 96 seeds per treatment) and extract or water, according to the treatment (Hernández et al., 2015a).

For the microbioassay, the extract was applied between two agar layers, similarly to the sandwich method described by Fuji (2003), Miyaura (2012) and Morikawa (2012) according to Hernández et al. (2015a), but replacing the six-well plastic culture plate of 10 mL by the removable reaction plate, where 100 µL of each of the two agar layers (0.75% w/v) were applied, between which the extracts were placed according to the respective concentration or water as a control. Finally, each well received a seed and the plates were incubated in moist chambers.

The traditional Petri dish culture technique served as comparison of microbioassay and was assembled as described by John et al. (2006), using Petri dishes (∅ 90 mm) and filter paper. The same amount of seeds (24) were placed on each plate and 3 mL of the extract or water was added to the filter paper (Figure 3). In both methods, the plates were maintained at room temperature (22 ± 2 °C) and diffuse natural light.

The number of germinated seeds was evaluated every 24 hours; a seed was considered germinated when its radicle emerged 1 mm or more. With these data, the Total Germination (G_T) and the Allelopathic Response Index (IR) were calculated at the end of the experiment:

Total germination (G_T): (Fernández et al., 2012)

$$G_T = [N_T \cdot 100] / N$$

N_T : Sprouted seeds for the treatment, at the end of the experiment.

N : Number of seeds to germinate.

Response Index (IR): (Williamson and Richardson, 1988; cited by Hu and Zhang, 2013).

IR = 1-(c/T) (T ≥ C) or IR= (T/c)- 1 (T< C)

T: total germination value or germination speed index in the treatment with extract of *I. batatas*.

C: value of seed germination variable in the control, with distilled water.

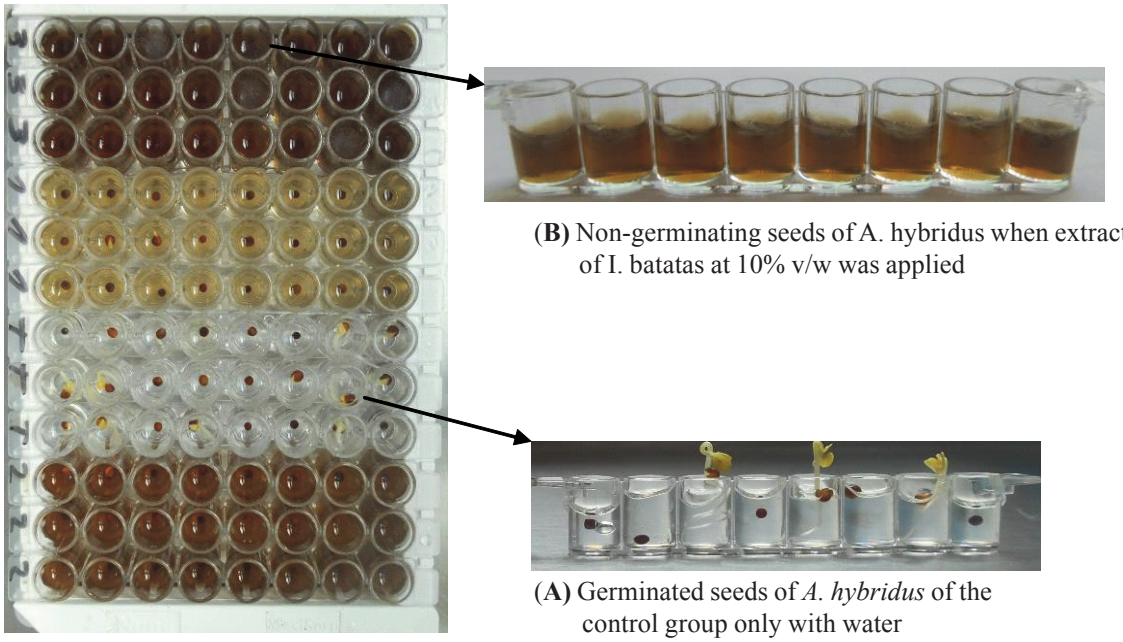
IR: A negative result indicates inhibition, a positive result indicates stimulation.

Statistical analysis

A completely randomized experimental design was used, with four replicates in each treatment. The data obtained were processed with the SAS statistical package. Simple analysis of variance, Shapiro-Wilk test for normality and Levene's test for homogeneity were applied. Differences between the two bioassays were estimated by the t-student test, while differences between treatments for each species were determined by the multiple proportions comparison test, using the Chi-square statistics. In each case, the analysis was performed with an error probability $p \leq 0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the plate where the test was performed with *A. hybridus* seeds and different concentrations of sweet potato extracts. Figure 1A displays a 8-well strip belonging to control group, with seeds immersed in the medium without the extract, where normal growth and normal germination were observed in each well. In contrast to this finding, when the strips with the seeds of the recipient species were observed in the medium containing the extract of *I. batatas* (10% w/v), they showed total inhibition of germination after 3-5 days of incubation (Figure 1B).



(**B**) Non-germinating seeds of A. hybridus when extract of I. batatas at 10% v/w was applied

(**A**) Germinated seeds of *A. hybridus* of the control group only with water

Below (A) the control treatment (water), where *A. hybridus* optimal germination and growth are observed. Above (B) a sample with the treatment using the *I.* batatas extract at 10%, where inhibition of seed germination is observed.

Figura 1 - Plate with the sandwich-type microbioassay development.

The amount of only 100 µL of medium placed in each well with the two layers of nutrient agar forms a sandwich, where the diffusion of the extract to be tested has a more closed link with the seed of the recipient species to be tested. This also avoids sudden changes that occur in the traditional Petri dish culture technique where the samples may become dehydrated or the concentrations of the extract under evaluation may alter. In the microbioassay, only 50-100 µL of the extract under assessment was used; compared with the 3 mL used in the bioassay, this is a very significant saving when the phytotoxic effect of extracts has to be measured, and also when there is a very small amount of extract or fractions obtained from a particular separation process.

In all the recipient species, the response index (IR) shows a clear reduction of germination, when the concentration of extracts of *I. batatas* increases. This differs from the Petri dish test according to the weed species and the concentration (Figure 2).

In the case of *P. oleracea*, when Petri dishes were used, the activity was almost nil with respect to the control, and an stimulation of more than 20% with the lowest concentration (1% w/v) was also noticed. However, the sandwich-type microbioassay revealed a reduction in germination between 16 and 60% and an inhibitory IR from 5% w/v. The other two recipient species displayed inhibitory responses with both methods, *A. hybridus* reaching an IR inhibition rate of 0.80 at the highest concentration of *I. batatas* extract.

In the case of *B. campestri*, concentrations of 1 and 5% w/v had a greater inhibition of germination, but in the end both methods showed with a very similar result, close to 1.0, with the highest concentration.

Thus, it may be summarized that the Response Indices (IR) found for both *P. oleracea* and *A. hybridus* showed a small difference with a negative value (IR) in the microbioassay, which detected the small variations produced when highly concentrated extracts of *I. batatas* were

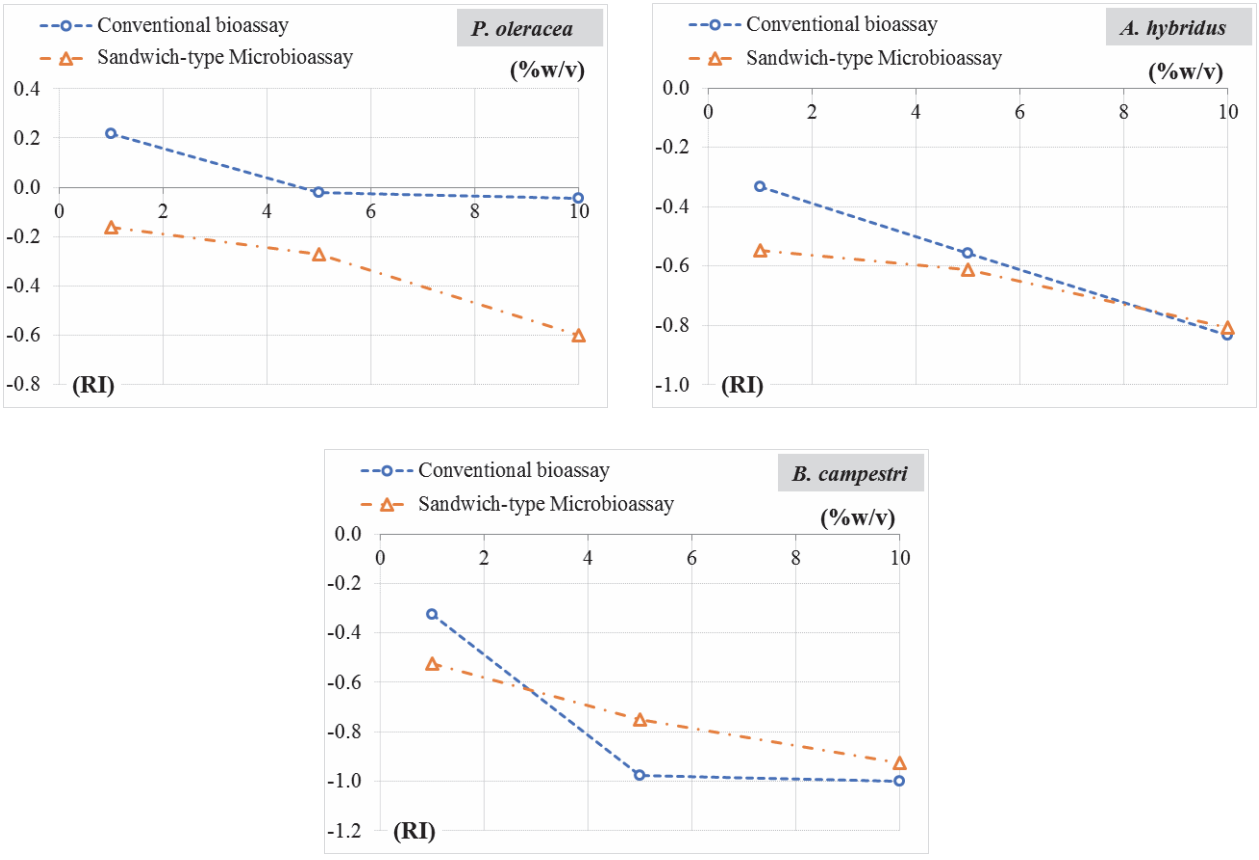


Figure 2 - Response Index (RI) of the three recipient species: *P. oleracea, A. hybridus* and *B. campestri,* at different concentrations of *I. batatas* extract.

applied every 24 h, in comparison with the traditional Petri plate method. The effectiveness of the test in Petri dishes can be altered when not properly protected, increasing the concentration of the extract by evaporation of the latter. This result, in seeds with longer germination periods, reduces the chances of germination by varying the concentration conditions of the allelochemicals and the osmolarity of the extract.

These findings were reported by John et al., 2006; Hernández et al., 2015a, who also added that the microbioassay plate has adequate aeration, which prevents the dehydration of the seeds during the germination process, especially if they are smaller-sized seeds and with a long incubation period.

This could assign a greater probative weight when determining the phytotoxic effect of a donor plant in comparison with the traditional Petri dish method, as it seems to be a very effective technique to measure inhibition levels when using *P. oleracea* and *A. hybridus*. Particularly, *B. campestri* was the most inhibited (53-100%) by the sweet potato extracts, becoming more effective when the concentration levels of the extracts were increased, followed by *A. hybridus* with 56-83%, and *P. oleracea* (16-60%).

These results corroborate the findings reported by Hu and Zhang (2013), who revealed the existence of a differential sensitivity with recipient species, when *Chenopodium ambrosioides* showed total resistance to aqueous extracts of leaves of *Chromolaena odorata*, while *A. spinosus* was affected by all the concentrations evaluated.

A shown in Table 1, when the effect of the extracts of *I. batatas* on the germination of the three weed species was statistically analyzed, an increased germination percentage of *P. oleracea* (57.3%) was observed in relation to *B. campestri* (41.8%) and *A. hybridus* (32.3%), which indicates that this species may be a good a model to determine allelopathic responses of plant extracts, both stimulatory and inhibitory, although this implies that the seeds must be previously evaluated to verify whether they germinate well, and then assess their sensitivity to the extracts (Oliveros-Bastida, 2008).

When comparing both tests with *P. oleracea*, the microbioassay revealed statistical differences between the control and the concentrations of 5 and 10% of the extract. The Petri plate method

Table 1 - Effects of *I. batatas* extract on the germination process of three different weed species: *P. oleracea*, *A. hybridus* and *B. campestri*

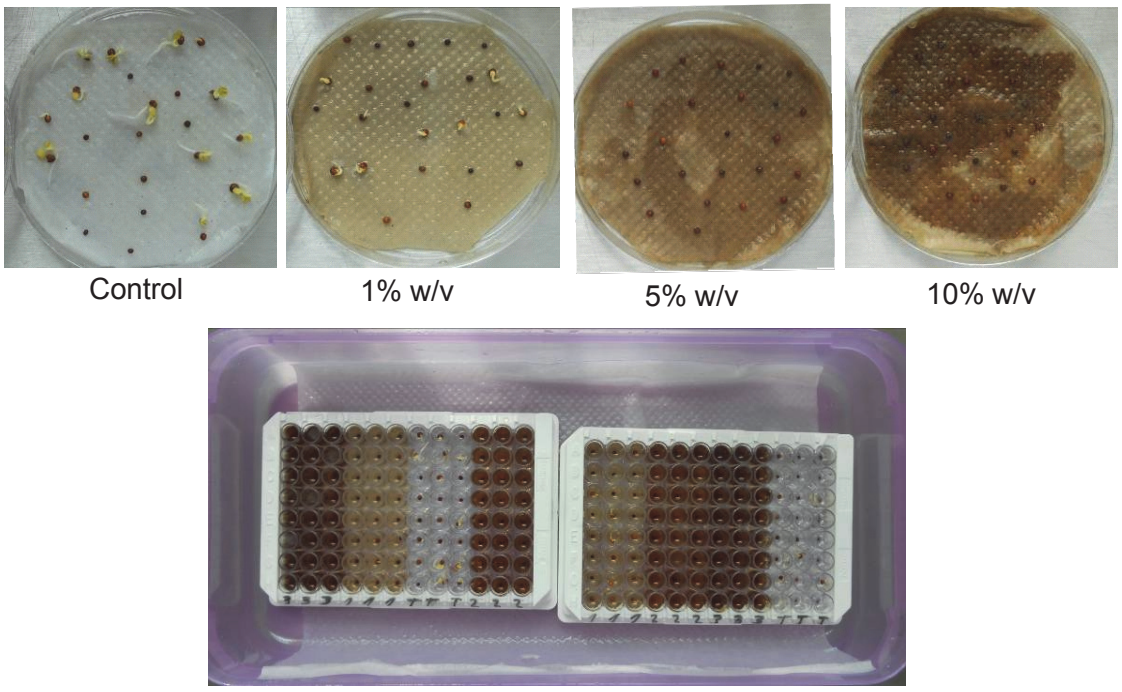
P. oleracea	Bioassay	Microbioassay	T student (p value)
Control	49.3 b	57.3 a	0.493
1 % w/v	62.5 a	47.8 ab	0.046
5 % w/v	48.3 b	42.0 b	0.540
10 % w/v	47.0 b	23.0 c	0.042
Chi-square/p value	6.0 / 0.010	24.64 / 0.000	
A. hybridus			
Control	18.75 a	32.29 a	0.041
1 % w/v	12.8 ab	14.5 b	0.755
5 % w/v	8.3 b	12.5 b	0.365
10 % w/v	3.0 c	5.0 c	0.488
Chi-square/p value	14.08 / 0.003	27.29 / 0.000	
B. campestri			
Control	41.5 a	41.8 a	0.980
1 % w/v	28.0 b	20.0 b	0.422
5 % w/v	1.0 c	10.3 c	0.206
10 % w/v	0	3.3 c	
Chi-square/p value	45.42 /0.000	24.64 / 0.000	

only brought out differences between the control and the concentration of 1% of the extract, with which the germination of this species was stimulated.

When applied on *A. hybridus*, the control did not show any difference in comparison with the extract at 1%, while germination was greatly decreased as the extract concentration increased. Similarly to what was observed when Petri dishes were used. When *B. campestri* was the recipient species, the control differed from all the extracts applied, displaying a gradual adverse effect on its germination, increasing the concentration of the *I. batatas* extract with 0 and 3 for Petri plates and microbioassay, respectively. This suggests a great similarity in terms of efficiency of both tests to detect the inhibitory activity of *I. batatas*. However, the sandwich-type method showed greater feasibility, less consumption of extracts or active fractions of the donor, less use of resources, reduced space, especially for small-sized seeds and long germination periods (John et al., 2006; Hernández et al., 2015a).

The sandwich-type microbioassay method definitively confirmed the phytotoxic potential of *I. batatas* on the weed species tested (Torres et al., 2003, Hernández, 2015, Hernández et al., 2015b), evidencing a reduction in seed germination and seedling growth. (Hu y Zhang, 2013).

Figure 3 shows the assay development in Petri dishes with different concentrations of extracts (1, 5 and 10% w/v) and under the same conditions applied on the 96-well plates, where similar germination values were observed, corroborating the results achieved by Hernández et al. (2015b). This figure exhibits the limited capacity of Petri dish testing to evaluate the seeds and the space that is required for their incubation, evidencing the need to constantly wet the paper to avoid dehydration during the testing period.



Note the lower number of seeds treated and the ample space required for incubation per plate. Under the same conditions tested with the microbioassay, 96-well plates and the small space needed to evaluate and repeat about 200 seeds of different receptor species.

Figure 3 - The image above shows the test with Petri dishes, determining the effect of three different extracts of *I. batatas* and one control on *A. hybridus*.

While the microbioassay requires a smaller area for its execution, but is capable of evaluating a larger number of samples, the conventional bioassay with Petri plates requires more space in the germination chamber; an additional benefit regarding the sandwich-type assay, which uses 6-well plates, even larger, and spends more medium than the microbioassay (Fujii et al., 2003; Fujii et al., 2004; Morita, 2005; Anjum et al., 2010; Takemura et al., 2013).

This method represents an obvious alternative that optimizes resources/analyses and requires a minimum amount of donor extract in laboratories with little infrastructure, in comparison with what has been exposed by Fuji et al. (2003) and Miyaura (2012). However, a standardization process to validate the efficacy of the method, with a larger number of species and types of recipient seeds, would provide reliable data on this new method.

Thus, it is concluded that the application of the sandwich microbioassay method allowed to efficiently determine the phytotoxic activity of the extracts of *I. batatas* on the recipient species *A. hybridus*, *P. oleracea* and *B. campestri*, with inhibitory (IR) responses of 56-83%, 16-60% and 53-100% respectively, percentages that increased according to the extract concentrations of 1-10% w/v. Of the three species evaluated with different concentrations of the *I. batatas* extract, the most responsive was *B. campestri*, which can be considered a good control for future tests, as it displayed acceptable percentages of germination and a negative inhibition index up to 100%. The use of extracts from *I. batatas* is a viable and environmentally friendly alternative, with a great potential for the Integrated Weed Management plan applied in farm crops geared towards a sustainable agriculture.

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