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Formulation of a bioherbicide with metabolites from *Phoma* sp.

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ABSTRACT

Weeds are one of the main problems faced by horticulturists, which are responsible for significant losses in many vegetable crops. The utilization of microbial bioproducts are an efficient alternative to weeds control. Aiming at replacing synthetic herbicides by bioherbicides and looking for strategies which are less aggressive to the environment, this work provides a bioproduct formulated with metabolites produced by *Phoma* sp. with herbicidal activity against bioindicated species of plants and weeds. Formulations containing a cells-free or spores-free culture filtrate, palm oil, Span® 80 and Tween® 80 were evaluated in order to increase the phytotoxicity. The best formulation contained 2.8% (w/v) surfactant (Span® 80 and Tween® 80), 8.2% (w/v) oil concentration and hydrophilic-lipophilic balance of 12.8. Combining adjuvants with culture filtrate of *Phoma* sp. showed phytotoxic efficiency against *Bidens pilosa*, *Amaranthus retroflexus* and *Conyza canadensis*.

1. Introduction

Weeds compete with crops for space, nutrients, water, and light, causing substantial economic loss (Wu et al., 2017). Weed management practices can be performed in a variety of ways, such as physical, chemical, biological and cultural controls (Hammermeister, 2016). The chemical control method has been the main tool used elsewhere, mainly due to large product supply, labor saving and quick operation. However, the use of chemical herbicides has caused environmental problems related to health of farmers and emergence of resistant weeds (Dayan et al., 2012; Diánez Martínez et al., 2016; McKeon and Brichta, 2014; Yang et al., 2014). Activities aimed at sustainable development favor the introduction of other methods to regulate weed infestation (Kołodziejczyk, 2015). Therefore, the search for new herbicides with safer toxicological and environmental profiles and with new modes of action has been increased (Dayan and Duke, 2014).

An alternative to the intensive use of chemical herbicide are substances produced by secondary metabolism of fungi (Daniel et al., 2018; Marinho et al., 2017; Taban and Saharkhiz, 2015), bacteria and plants, which are being used naturally or serving as a model for the synthesis of new herbicides molecules (Dayan et al., 2009). Phytopathogenic fungi are one of the most studied groups in relation to their herbicide potential, since they have the specific ability to produce toxic substances. Furthermore, they are able to penetrate the leaves of plants, disintegrate their cellular structure and induce the production of necrotic lesions or chlorotic halo, with the advantage of being non-toxic to mammals and easily degraded in the environment (Li et al., 2003; Tebeest, 1991). The use of bioherbicides is an important step towards sustainability in agriculture (Cordeau et al., 2016).

Some studies have demonstrated that toxins produced by phytopathogens express herbicidal activity (Bastos et al., 2017; Brun et al., 2016; Javaid and Ali, 2011; Pes et al., 2016). *Phoma sp.* is one important microorganism used for producing metabolites with biological control of weeds, causing naturally lesions on leaves, stalks, flowers, and pods, as well discoloration of the hypocotyl, cotyledon, and roots (Boerema et al., 2004Boerema et al., n.d.). Studies demonstrated important outcomes using fungus of genus *Phoma* sp. as an herbicidal agent in target plants and some weeds species (Bailey et al., 2011; Brun et al., 2016; Graupner et al., 2006; Todero et al., 2018).

However, one challenge in the development of bioherbicides is their low herbicidal activity. In general, the low effect occurs because the fungal phytotoxins are usually found in very low concentration in culture media (Varejão et al., 2013). Alternatives to overcome this difficulty require synthetic modifications to create an effective product (Sica et al., 2016) or the use of an appropriate combination of adjuvant in a formulation to increase the herbicidal activity, as these are

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intended to modify or reinforce the physic-chemical characteristics of the solution (Rana and Rana, 2016). Some studies in the scientific literature demonstrated that the formulation of microbial metabolites increased the herbicidal activity of product (Bastos et al., 2017; Piyaboon et al., 2016; Sica et al., 2016).

Based on these aspects, this study has focused on the formulation of a bioherbicide with secondary metabolites produced by *Phoma* sp., which helps horticulturists to produce organic products. The study was divided into two stages: (1) evaluation of different formulations containing a cells-free or spores-free culture filtrate, palm oil, Tween® 80 and Span® 80 to increase phytotoxicity against a bioindicator plant (*Cucumis sativus*); (2) evaluation of the best formulation for controlling of *Bidens pilosa*, *Amaranthus retroflexus* and *Conyza canadensis*.

2. Material and Methods

2.1. Production of bioherbicide

The strain of *Phoma* sp. (NRRL 43879) was maintained in a potato dextrose agar (PDA) at 4-6 °C and subcultured every 15 days. For the inoculum, two 6 mm disks of fungal mycelium were taken from a plate subcultured for 7 days at 28 °C in a bacteriological oven and transferred to a Erlenmeyer flask containing 125 ml of culture medium with the following composition (g/l): potato (200), dextrose (20), peptone (10), yeast extract (7.5), $(NH_4)_2SO_4$ (2), $FeSO_4.7H_2O$ (1) and $MgSO_4$ (0.5) (Parra et al., 2005; Zhang et al., 2012). The flasks were maintained at 28 °C and 120 rpm for 7 days (Innova 44R, New Brunswick).

The biomass was separated from the fermentation broth by filtration using a filter paper (Whatman, number 2, Brazil) followed by centrifugation (Eppendorf, model 5804R, Germany) at 10,000 rpm for 10 min, with relative centrifugal force of 5.320 × g. The supernatant was concentrated to 30% (v/v) through membrane separation process composed of a feed reservoir totalizing 500 mL of crude fermented broth free of cells, a peristaltic pump (Tecnal, model TE-198, Brazil), a tubular glass reservoir of 1 L, two pressure gauges, a tubular module containing a polyvinylidene difluoride (PVDF) hollow fiber membrane (Microza, model UMP 1047R, Japan) for microfiltration, valves and reservoirs. The microfiltration tubular module was formed of 0.2 µm of nominal pore size and 0.09 m^2 of filtration area. The pump was started and the following conditions were set: working pressure of 10^5 Pa, mass flow rate of 50 mL/min and temperature of 25 °C. The concentration of biomolecules started when the system reached the steady-state condition. Only retentate was used in the formulations.

2.2. Bioherbicide formulation and characterization of emulsion

For the preparation of emulsions, a mixture of two surfactants Span® 80 and Tween® 80 with different hydrophilic-lipophilic balance (4.3 and 15, respectively) and palm oil was performed in order to evaluate the efficiency of adding adjuvants in the culture filtrate of *Phoma* sp. Span® 80 and palm oil (mixture A) were homogenized in an Ultra-Turrax equipment at 7000 rpm for 1 min to obtain a homogeneous emulsion. The culture filtrate was blended with Tween® 80 for 1 min (mixture B). Thereafter, the mixture A was gradually added to the mixture B and kept under stirring at 7000 rpm for 5 min, totalizing an emulsion mass of 10 g. The effects of oil concentration (1-10%, w/w), emulsifier concentration (1-10%, w/w) and hydrophilic-lipophilic balance (HLB) (4.3-15) were evaluated on the phytotoxicity based on a central composite rotational design (CCRD). The mass of adjuvants (palm oil, Span® 80 and Tween® 80), distilled water and culture filtrate is presented in Table 1.

2.2.1. Density and pH

The densities of formulations were measured in triplicate using the DDM 2911 Plus digital density meter (Rudolph Research Analytical, USA) by touchscreen at 20 °C. The samples were inserted into the device through a syringe, using 3 mL of each sample. The pH was measured using a bench pH meter at 25 °C. Measurements were done in triplicate for all spraying liquids and water.

Table 1
CCRD matrix for the formulations of bioherbicide using different concentrations of metabolites, palm oil, Span®80 and Tween®80.

Formulation	Palm oil (g)	MS (g)	MT (g)	HLB (-)	CF (g)
Control A - distilled water	-	-	-	-	-
Control B – culture filtrate from *Phoma* sp.	-	-	-	-	10.0
01	0.28	0.2224	0.0576	6.5	9.4
02	0.82	0.2224	0.0576	6.5	8.9
03	0.28	0.6514	0.1686	6.5	8.9
04	0.82	0.6514	0.1686	6.5	8.4
05	0.28	0.0576	0.2224	12.8	9.4
06	0.82	0.0576	0.2224	12.8	8.9
07	0.28	0.1686	0.6514	12.8	8.9
08	0.82	0.1686	0.6514	12.8	8.4
09	0.1	0.2724	0.2775	9.7	9.4
10	1	0.2724	0.2775	9.7	8.5
11	0.55	0.0495	0.0504	9.7	9.4
12	0.55	0.4953	0.5047	9.7	8.5
13	0.55	0.5500	0.0000	4.3	8.9
14	0.55	0.0000	0.5500	15	8.9
15	0.55	0.2724	0.2776	9.7	8.9
16	0.55	0.2724	0.2776	9.7	8.9
17	0.55	0.2724	0.2776	9.7	8.9

MS = Mass of Span®80
MT: Mass of Tween®80
HLB: Hydrophilic-lipophilic balance
CF: Mass of culture filtrate

Table 2
Description of concepts applied to toxicity assessments according to SBCPD. Source: (SBCPD, 1995).

%	Description
0 – 20	No injury or effect
20 – 40	Slight injury and/or reduction of growth with rapid recovery
40 – 60	Moderate injury and/or reduction of growth with slow recovery or definitive
60 – 80	Severe injury and/or reduction of non-recoverable growth and/or reduction of the stand
80 – 100	Complete destruction of crop or only a few live plants

2.2.2. Surface tension

The surface tension was measured in triplicate using a KRÜSS DSA25 drop form analyzer (KRÜSS, Germany) at 25 °C and determined with the dropping drop method (10 drops per solution of formulations).

2.3. Bioassays

The bioassays were carried out on two-stage of post-emergence of plants: initially, preliminary tests were carried out on *Cucumis sativus* plants to determine suitable formulation conditions. After this optimization, bioassays were carried out in the best condition using three different weeds, *Bidens pilosa*, *Amaranthus retroflexus* and *Conyza canadensis*.

2.3.1. Preliminary bioassay on Cucumis sativus

The herbicidal activity of secondary metabolites from *Phoma* sp. was determined through the application of different CCRD assays in *Cucumis sativus*. Although it is not a weed, this species was studied because it is normally used in tests with chemical herbicides. Seeding was done in plastic cups (180 mL) containing the commercial substrate Mecplant®, being cultivated for 7 days in a greenhouse at 25 °C and 70% relative