

Production and formulation of a bioherbicide as environment-friendly and safer alternative for weed control

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ABSTRACT

Bioherbicides are an alternative more environment-friendly for weed management in organic agriculture and also providing new modes of action for conventional agriculture. In this way, the present study is focused on the optimization of bioherbicide production by *Phoma* sp. in submerged fermentation and the development of a formulation to increase its efficiency. Fermentation media based on sucrose and corn steep liquor (CSL) was optimized aiming to maximize the target control. Additionally, several formulations containing adjuvants and fermented broth were developed aiming to increase the herbicidal activity. The optimized condition for bioherbicide production was 13 g.L⁻¹ of sucrose and 15 g.L⁻¹ of CSL. The use of adjuvants increased the herbicidal activity in pre and post-emergence of *C. sativus* and *S. bicolor*. The maximum herbicidal activity was obtained with 30% v/v of fermented broth combined with 1% v/v of surfactant and nitrogen source.

Keywords: *Natural herbicide; industrial fermentation; microbial source;*

1. INTRODUCTION

Ensuring global food security with a changing environment and shrinking natural resources are the major challenges in the present era. Population density has risen rapidly over the last few decades, and supplying food to such a growing population is a challenge all over the world. Therefore, sustainable use of natural resources in the face of high population density is critically important, and, consequently, food insecurity is overwhelming specially for developing countries [1].

Since the introduction of chemical herbicides in the 1940s, some limitations as weeds resistant to herbicides, soil contamination and water resources and hazardous chemical waste to non-target organisms are evident, it is important to search for new technologies or new molecules to overcome these problems. Among the alternatives is the search for natural products, such as the use of phytotoxic secondary metabolites of microorganisms, which could serve as natural herbicides for application or discovery of new molecules with potential herbicide [2-7]. The appeal for use of bioherbicide in agriculture is also the growing need for new products with safer toxicological and environmental profiles. Natural product-based herbicides are considered by the public to be generally safer than conventional, synthetic herbicides. In general, the half-life of bioherbicides molecules are usually shorter than that of chemicals, so they are expected to be more environment-friendly [8].

Research involving secondary metabolites as a source of natural herbicides has been demonstrating many characteristics, among them, the possibility to discover novel mechanisms of action. Thus, new biochemical bioherbicides have the potential for greatly improving weed management in organic agriculture and providing new modes of action for conventional agriculture [9].

Microorganisms are important in this kind of research through the production of a large number of bioactive secondary metabolites by fermentation processes or by derivation of their compounds.

One of the most studied genera with potential bioherbicide production is *Phoma*, due to the diversity of species that produce phytotoxic metabolites [6, 10-14]. *Phoma macrostoma*, for example, is a pathogen that causes chlorosis, leaf spot and necrosis in woody and herbaceous plants, causing black rot of artichoke leaves due to the presence of a phytotoxic secondary metabolite known as macrocidins molecules [15-19].

The preferred cultivation procedure is by submerged fermentation, where various factors, mainly medium composition, affect the production of phytotoxins (Varejão et al., 2013). Definition of medium composition is often considered as a major component in the cost of fermentation products, which normally responds to almost 50% of the production process [20,21]. Agro-industrial wastes are a good alternative to reduce the cost of fermentation media. However, special attention should be given for the purification steps of phytotoxins, where the complex composition of media may difficult the purification [22].

The efficiency in the application of crop protection products, for example, synthetic or natural herbicides is related to drift potential, loss by draining and target coverage. It is a complex process influenced by several factors such as the type of spray equipment used and the physicochemical properties of the broth [23,24]. The action of crop protection products is dependent on the spray mixture constituents, which usually consist of product, water and adjuvants. Generally, adjuvants enhanced the application effectiveness of products to the field [25]. The adjuvant interaction and phytosanitary product is a complex process involving physical,

chemical and physiological properties, and changes for each condition tested [26]. Adjuvants act differently from each other, affecting the wetting, sticking and spreading, foaming and dispersion of the spray solution [27,28]. Based on these aspects, the main objective of this work was to optimize the media composition

2. MATERIALS AND METHODS

2.2. Materials.

Corn steep liquor (CSL) was obtained from Ingredion (Mogi Guaçu, SP, Brazil), and Sucrose (Cristal) was purchased in a local market. Other chemicals, namely, $(\text{NH}_4)_2\text{SO}_4$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, MgSO_4 were purchased from Sigma-Aldrich. Silwet L-77 (FMC) and APORTE Plus were purchased in a local agricultural supply store.

2.3. Microorganism, inoculum and fermentations.

Phoma sp. (NRRL 43879) was obtained at the Agricultural Research Service Culture Collection. The culture was maintained in a potato dextrose agar (PDA) between 4°C and 6°C and subcultured every 15 days. Cell production for pre-inoculum was incubated on PDA in a Petri dish for 8 days at 28°C. The inoculum was cultivated in Erlenmeyer containing 50 mL of fermentation medium at 28°C, 120 rpm for 5 days in an orbital shaker (Innova 44R, New Brunswick Scientific). The media composition was (g.L⁻¹): glucose (20.0), peptone (10.0), yeast extract (7.5), $(\text{NH}_4)_2\text{SO}_4$ (2.0), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.0) and MgSO_4 (0.5) (Klaic et al. 2016).

2.4. Optimization of media composition for bioherbicide production.

The fermentation media was composed of sucrose and corn steep liquor (CSL) as main substrates supplemented with mineral salts (g.L⁻¹): $(\text{NH}_4)_2\text{SO}_4$ (2.0), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.0) and MgSO_4 (0.5). The initial pH was adjusted to 6.0. The fermentations were inoculated with 10% (v/v) of inoculum and were carried out in Erlenmeyers containing 125 mL of fermentation medium at 28°C, 120 rpm for 7 days (Innova 44R, New Brunswick Scientific). After the fermentations, cells were separated by centrifugation at 10.000 rpm for 10 minutes (Eppendorf, model 5810R), supernatant was filtered using a 0.45 µm PVDF membrane. The filtered sample without any chemical addition was used in the bioassay. In this step, the bioassays were carried out in pre-emergence and analysis of seedling growth of *Cucumis sativus*.

A central composite rotatable design (CCRD) for two independent variables was proposed to optimize the concentrations of sucrose and CSL in the bioherbicide production by *Phoma* sp. The range of variables investigated was 15-25 g.L⁻¹ for sucrose and 5-15 wt% for CSL. At this step, the responses evaluated were the pre and post-emergence herbicidal activity in the bioassay using cucumber (*Cucumis sativus* L. variety wisconsin) as the target plant.

2.5. Emulsion containing the bioherbicide.

Different formulations of fermented broth optimized in CCRD with surfactant were prepared to evaluate the bioherbicide activity. The formulation consisted in an emulsion containing different concentrations of fermented broth (4.8 to 55.2 % v/v), surfactant (Silwet L-77 – 0.0 to 2.0 % v/v) and one nitrogen source (APORTE Plus – 0.0 to 2.0 % v/v). A CCRD for three independent variables was conceived to optimize the concentration of each component in the emulsion. The responses evaluated were pH,

for bioherbicide production by *Phoma* sp. in submerged fermentation. In addition, several formulations containing adjuvants and fermented broth were developed aiming to increase the herbicidal activity.

surface tension, emulsion stability, pre and post-emergence activities towards cucumber (*Cucumis sativus* – dicotyledonous) and sorghum (*Sorghum bicolor* – monocots).

The experimental apparatus to produce the emulsions was composed of a flow cell made of glass with cooling by circulating water in the jacket and working volume of 250 mL, a thermostatic water bath (temperature accuracy of ± 1.0 °C) for temperature control, a high-intensity ultrasound processor of 400W and frequency of 24 kHz (Hielscher, Model UP 400S). The ultrasound was equipped with a titanium probe diameter 22 mm and length 200 mm (Model H22L2D). The components (fermented broth, surfactant and nitrogen source), at specified concentrations, were added in the cell flow and the final volume adjusted to 100 mL using distilled water. The temperature was adjusted to 30 °C ± 2 °C by circulating water through the jacket and the sample sonicated for 2 h. The emulsion was maintained at rest for 30 minutes and, after, used for pH, surface tension and herbicidal activity determination.

2.6. Bioassays.

2.6.1. Pre-emergence

The germination tests were carried by applying 10 mL of fermented broth in a germitest paper containing 25 seeds of each culture and maintained at 25°C (POL-EKO, model KK 350). Control test was carried out replacing fermented broth by culture media. The seeds germination was evaluated at 4 (first count) and 10 (second count) days after the application of bioherbicide, according to Brazilian rules for seeds analysis. Afterward, the germinated or non-germinated seeds were counted. All seeds that presented the primary root protrusion higher than 2 mm were considered as germinated. For seeds that germinated, an analysis of seedling growth was conducted. After 72 hours, the length of each seedling (radicle elongation and hypocotyl) was measured with the aid of a caliper. Each treatment was replicated four times.

2.6.2. Post-emergence.

The seeds used in the experiment were obtained from local market and did not suffer any treatment before the seeding. Three seeds of each culture (*C. sativus* and *S. bicolor*) were sown in separated propylene vessels of 200 mL with commercial substrate (Macplant®) without any treatment. After the emergence, only one plant was maintained, being cultivated for 7 days in a greenhouse located at the Federal University of Santa Maria. The bioherbicide (formulated or not) was applied by using a backpack sprayer pressurized by CO₂, provided with a bar pattern with four tips model Teejet XR 110.02 with pressure of 40 lbf and spacing tips of 0.5 m. The travel velocity was 1 m.s⁻¹ and the volume of liquid was 200 L.ha⁻¹. The applications were carried out when the plants presented 2-3 leaves. Five days after de application plant injury was visually estimated as percent growth reduction by comparison to untreated controls, where 100% represents complete plant death and 0% represents no effect [29].

2.7. Physical-chemical analysis in the emulsion.

The pH was measured using a pHmeter calibrated at temperature of 25 °C. The surface tension was determined based on the Du Nouy ring method [30] using a tensiometer (Kruss, model K6). The test consisted of measuring the tension experienced by the ring at the end of a flexible rod, placed on the sample surface and pressed against it until it undergoes repulsion. Tensiometer was calibrated with milli-Q water. Density was determined in a density meter (Anton Paar, model DM 4500 M) by direct injecting an

aliquot (1 mL) of supernatant at 25°C. The stability of emulsion was evaluated visually by analyzing the formation of phases after 30, 60, 360, 600, 720 and 1440 min.

2.8. Statistical analysis.

All statistical analysis was carried out using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 95%.

3. RESULTS

3.1. Optimization of fermentation media to maximize the herbicidal activity.

Table 1 presents the results referring to herbicidal activity in pre-emergence and seedling growth of *C. sativus* obtained in 11 runs of the CCRD plus two control tests. The herbicidal activity and the seedling growth were not affected by the application of distilled water or culture medium (control tests). In this way, the results obtained in the runs of CCRD are due to the presence of metabolites produced by the fungus, since there is a great variation in both responses among the runs. The highest herbicidal activity and the lowest seedling growth were 91% and 0.9 cm, respectively, obtained in run 3. The intermediary herbicidal activity was obtained in runs 4, 5, 6 and 8, whereas in other runs the results were little expressive. However, it is important to point out that even in the runs with low herbicidal activities, the seedling growth was severely affected by the metabolites produced by the fungus. For example, runs 1 and 2 presented very low herbicidal activity, but the seedling growth was about one-third of control.

These results are corroborating the hypothesis that *Phoma* sp. may be used for bioherbicide production. Also, it is demonstrated that the herbicidal power of fermented broth is dependent on media composition and the use of experimental design tool is a good strategy to find an ideal composition to maximize the herbicidal activity of broth. For this reason, data of Table 1 were used to estimate the parameters of a quadratic model expressing the herbicidal activity in pre-emergence in the function of independent variables. Eq. 1 presents the significant terms ($p < 0.05$) of the model:

$$HA = 32.72 - 7.94 \cdot S + 12.05 \cdot S^2 + 32.50 \cdot CSL \quad (1)$$

where HA is the herbicidal activity of fermented broth (%), S and CSL are the coded values of sucrose and corn steep liquor concentrations, respectively.

The model was validated by ANOVA, since the calculated F-test was about six times greater than the tabled one and the coefficient of determination (R^2) was 0.9588. The linear terms are indicating that the increase of sucrose concentration from level -1

to +1 reduces the herbicidal activity, whereas the increase of CSL concentration has an opposite behavior. However, it is necessary to define the medium composition that maximizes the production of molecules of interest. For this reason, Eq. 1 was used to express the herbicidal activity in the function of independent variables, which is expressed in Figure 1. Maximum herbicidal activity was obtained at sucrose and CSL concentration of 13.0 and 15.0 g.L⁻¹, respectively. This result was confirmed after the realization of one additional fermentation, where herbicidal activity in pre-emergence was 93%. At this condition, the process was considered optimized. All fermentations from this part were carried out using 13.0 and 15.0 g.L⁻¹ for sucrose and CSL concentrations, respectively.

3.2. Formulation of bioherbicide.

Table 2 presents the physical-chemical parameters and herbicidal activity in pre and post-emergence of *C. Sativus* and *S. biocolor* for the 17 runs of the CCRD. Viscosity, density and pH presented little variation among the runs, presenting similar values of the crude fermented broth. Surface tension values for the fermented broth and water were 45 and 69 mN m⁻¹, respectively.

The bioassays were verified differences among the runs in pre and post-emergence for both species. The seeds germination of *C. sativus* was completely inhibited for all runs, whereas in post-emergence the herbicidal activity ranged from 57% to 100%. For *S. bicolor* there was great variability in the pre and post-emergence activities, but full inhibition of germination or total death of plants was obtained. The best efficiencies in control for both monocots and dicotyledonous species were found in the tests 10, 12, 14 and central points where 100% of control was achieved for all species. At this condition, formulation was composed for 30% (v/v) of fermentation broth, 1% of surfactant and nitrogen source, obtaining efficiency higher than the use of pure fermented broth on control. Figure 2 presents the comparison of post-emergence activity of control test (using distilled water) and treatments using 30% v/v of fermented broth combined with 1% v/v of surfactant and nitrogen 13 days after the application.

Table 1. Matrix of the CCRD design to evaluate the influence of media composition on herbicidal activity in pre-emergence and seedling growth of *C. sativus*.

Run	Sucrose (g.L ⁻¹)	CSL (%)	Herbicidal activity in pre-emergence (%)	Seedling growth (cm)
1	15 (-1)	5 (-1)	7.0 ^g	3.2 ^{cdef}
2	25 (1)	5 (-1)	1.0 ^g	5.0 ^{cd}
3	15 (-1)	15 (1)	91.0 ^a	0.9 ^f
4	25 (1)	15 (1)	59.0 ^{bcd}	1.0 ^f
5	13 (-1.41)	10 (0)	72.0 ^{abc}	1.4 ^{ef}
6	27 (1.41)	10 (0)	54.0 ^{cde}	5.4 ^c
7	20 (0)	3 (-1.41)	0.0 ^g	9.9 ^b
8	20 (0)	17 (1.41)	83.0 ^{ab}	1.9 ^{def}

9	20 (0)	10 (0)	31.0 ^f	5.0 ^{cd}
10	20 (0)	10 (0)	34.0 ^f	4.4 ^{cde}
11	20 (0)	10 (0)	33.0 ^f	3.7 ^{cdef}
C	--	--	0.0 ^g	13.3 ^a
F.B.*	13	17	3.0 ^g	12.6 ^a

*F.B.= fermentation broth, C= control without application of fermented broth. ¹Averages followed by the same letter in the column do not significantly differ by the Tukey test (p<0.05).

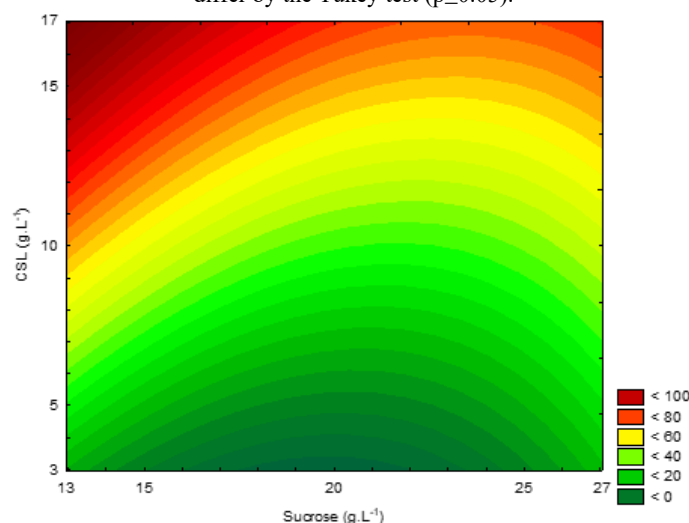


Figure 1. Contour plots expressing germination assay (a) cucumber seeds in function of sucrose and CSL concentrations.

Table 2. Matrix of the CCRD design to evaluate the influence of independent variables on the biological and physical-chemical parameters of the formulated bioherbicide.

Runs	X ₁ (% v/v)	X ₂ (% v/v)	X ₃ (% v/v)	pH	Surface tension (mN.m ⁻¹)	Viscosity at 25°C (mPa.s)	Density at 25°C (g.cm ⁻³)	<i>C. sativus</i>		<i>S. bicolor</i>	
								Pre-emergence	Post-emergence	Pre-emergence	Post-emergence
1	15 (-1)	0.4 (-1)	0.4 (-1)	5.3	20.4 ^d	1.4	0.9986	100.0 ^a	57.0	95.0 ^b	45.1
2	45 (1)	0.4 (-1)	0.4 (-1)	5.5	20.5 ^d	1.2	1.0022	100.0 ^a	95.3	85.0 ^c	87.0
3	15 (-1)	1.6 (1)	0.4 (-1)	5.1	20.6 ^d	1.4	1.0052	100.0 ^a	79.8	85.0 ^c	74.6
4	45 (1)	1.6 (1)	0.4 (-1)	5.4	20.5 ^d	1.2	1.0160	100.0 ^a	97.9	95.0 ^b	95.3
5	15 (-1)	0.4 (-1)	1.6 (1)	5.4	20.3 ^d	1.6	1.0107	100.0 ^a	81.9	80.0 ^d	77.7
6	45 (1)	0.4 (-1)	1.6 (1)	5.8	20.4 ^d	1.4	1.0194	100.0 ^a	100.0	85.0 ^c	98.4
7	15 (-1)	1.6 (1)	1.6 (1)	4.7	20.4 ^d	1.6	1.0052	100.0 ^a	89.6	100.0 ^a	85.0
8	45 (1)	1.6 (1)	1.6 (1)	5.4	20.5 ^d	1.4	1.0174	100.0 ^a	100.0	100.0 ^a	99.0
9	4.8 (-1.68)	1.0 (0)	1.0 (0)	4.7	20.3 ^d	1.8	1.0067	100.0 ^a	67.9	95.0 ^b	45.1
10	55.2 (1.68)	30 (0)	1.0 (0)	5.3	20.5 ^d	1.4	1.0188	100.0 ^a	100.0	100.0 ^a	100.0
11	30 (0)	0 (-1.68)	1.0 (0)	5.2	20.4 ^d	1.4	1.0029	100.0 ^a	95.3	100.0 ^a	95.0
12	30 (0)	2.0 (1.68)	1.0 (0)	5.3	20.3 ^d	1.5	1.0217	100.0 ^a	100.0	100.0 ^a	100.0
13	30 (0)	1.0 (0)	0 (-1.68)	5.8	25.1 ^c	1.0	1.0042	100.0 ^a	97.4	100.0 ^a	93.8
14	30 (0)	1.0 (0)	2.0 (1.68)	5.1	20.2 ^d	1.5	1.0105	100.0 ^a	100.0	100.0 ^a	100.0
15	30 (0)	1.0 (0)	1.0 (0)	5.3	20.1 ^d	1.4	1.0710	100.0 ^a	100.0	100.0 ^a	100.0
16	30 (0)	1.0 (0)	1.0 (0)	5.0	20.3 ^d	1.4	1.0170	100.0 ^a	100.0	100.0 ^a	100.0
17	30 (0)	1.0 (0)	1.0 (0)	5.4	20.1 ^d	1.5	1.0770	100.0 ^a	100.0	100.0 ^a	100.0
Water					69.2 ^a		1.0790	0.0 ^c	0.0 ^e	0.0 ^c	0.0 ^c
X ₁	100				45.2 ^b		1.0760	93.0 ^b		70.0 ^d	25.0

X₁ – Fermented broth; X₂ – Nitrogen source; X₃ – Surfactant. ¹Averages followed by the same capital letter in the column and lowercase letter in the line, do not significantly differ by the Tukey test (p<0.05).

3.3. Discussion.

Some microorganisms have a very low amount of their secondary metabolites, making it difficult for their industrial production. Thus, it is important to use simple methods as an optimization of the specific culture medium, allowing the formation of a significant increase in the production yield of the secondary metabolites [31]. In this context, the study demonstrates, through the control and root growth responses, the influence of the culture medium on production of secondary metabolites with herbicidal activity.

The production of molecules having herbicidal activity is influenced negatively by the sugar concentration in a mechanism

known as catabolite repression, which allows microorganisms to adapt quickly to a preferred (rapidly metabolisable) carbon and energy source first (in this work was sucrose). This is usually achieved through inhibition of synthesis of enzymes and molecules involved in catabolism of carbon sources other than the preferred one [32]. Klaic et al., 2017 [33] optimized the culture medium through agro-industrial residues such as bagasse, soybean meal and corn maceration water in the solid-state fermentation of the fungus *Phoma* sp., noted a significant influence of the culture medium on the phytotoxicity application of the bioherbicide. Certain secondary metabolites, such as xylanase, showed an increase in production when optimized for its culture medium in a study carried out with

the *Aspergillus niger* fungus [34]. Several studies have demonstrated the importance of studying the ideal culture medium to increase the production of the desired molecules, helping the possible industrial use of this process [35].

After being optimized the fermentation of the bioherbicide to the greater power of the herbicide activity, it sought to increase the efficiency of the bioherbicide through the use of adjuvants. It was observed through the results of this worked an influence of the application adjuvants with bioherbicide. In a study to improve the efficiency of a bioherbicide produced by the solid-state fermentation of the fungus *Diaporthe* sp., were used different combinations of Tween 80 and Span 80. Results showed that the use of adjuvants increased by three times its efficiency.[36]. Adjuvants are defined as any substance present in a formulation or added to the spray tank having the feature of modifying the biological activity or the application characteristics [37].

The main physicochemical properties related to phytosanitary products that are sensitive to changes caused by adjuvants are surface tension, viscosity, density, electrical conductivity and pH. In the present paper was showed the influence

of the adjuvants on viscosity, density and pH in the different formulations was observed. The surface tension presented a significant difference when we compared the values of the fermented broth with the use of the organosiliconated surfactant and the nitrogenated, respectively, 45, 20 and 25 mN m⁻¹. These values of fermented broth for the application of plant protection products have low retention capacity when applied to the cuticle of plants [27]. The result obtained here for surface tension corroborated with Iost et al. [37] studies, which tested six commercial surfactants in aqueous solutions, verifying the tendency of Silwet L-77 to reduce the surface tension close to 20 mN m⁻¹.



Figure 2. Visual effect of herbicidal activity obtained at formulation of run 14.

4. CONCLUSIONS

In this work, the culture media for bioherbicide production by the fungus *Phoma* sp. was optimized. The optimized condition for bioherbicide production was using fermentation media containing 13 g.L⁻¹ of sucrose and 15 g.L⁻¹ of CSL, obtaining 93% of bioherbicide activity in pre-emergence. The use of adjuvants

increased the herbicidal activity in pre and post-emergence of *C. sativus* and *S. bicolor*. Total inhibition of seed germination and total death of plants of *S. bicolor* and *C. sativus* were obtained with 30% v/v of fermented broth combined with 1% v/v of surfactant and nitrogen.

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6. ACKNOWLEDGEMENTS

The authors thank CAPES for the scholarships as well as FAPERGS and CNPq for the financial support for this work.



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