

Article

Microbial Prospecction for Bioherbicide Production and Evaluation of Methodologies for Maximizing Phytotoxic Activity

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Abstract: The occurrence of several weeds in crops of high economic value directly affects grain yield. This scenario led to the mass investigation of chemical products that circumvent these adversities and provide control potential. Nonetheless, the excessive application of chemical herbicides has generated significant concerns about the environment. Accordingly, the adoption of alternative practices, such as the application of microbial metabolites, emerges as strategic control actions, having a sustainability bias and allowing the reduction of risks of human and animal contamination. Appropriately, this study proposed to conduct a microbial prospecction of microorganisms capable of producing secondary metabolites to inhibit growth and generate phytotoxicity in weeds. Furthermore, to increase the herbicidal activity, different strategies were evaluated involving microbial co-cultivation and ultrasound-assisted extraction after fermentation. Accordingly, 63 microorganisms were isolated from weeds with disease symptoms and submitted separately to submerged fermentation. Initially, the bioherbicidal activity was evaluated in *Cucumis sativus* plants, and the most promising were applied in *Amaranthus hybridus* and *Echinochloa crusgalli*. Treatments with fermented broth obtained from co-cultivation and ultrasound-assisted extraction after the fermentation process indicated an inhibition of plant development. The most promising strains were A14.2 and B22.2 (*Nigrospora sphaerica*), B14 (*Bacillus velezensis*), and γ (*Aspergillus flavus*).

Keywords: *Amaranthus hybridus*; bioprospecting; bio-based products; *Echinochloa crusgalli*; potential phytotoxicity



Citation: Schein, D.; Santos, M.S.N.; Schmaltz, S.; Nicola, L.E.P.; Bianchin, C.F.; Ninaus, R.G.; Menezes, B.B.d.; Santos, R.C.d.; Zabot, G.L.; Tres, M.V.; et al. Microbial Prospecction for Bioherbicide Production and Evaluation of Methodologies for Maximizing Phytotoxic Activity. *Processes* **2022**, *10*, 2001. <https://doi.org/10.3390/pr10102001>

Academic Editors: Weiming Tan and Youhua Wang

Received: 30 August 2022

Accepted: 30 September 2022

Published: 4 October 2022

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1. Introduction

Weeds are one of the main problems in agriculture [1,2]. They have a high capacity to compete for resources such as micronutrients, light, and chemical and biological resources [3]. The weed species of the genera, *Amaranthus* sp., *Conyza* sp., *Echinochloa* sp., and *Bidens* sp., have been reported to cause damage to different important crops worldwide [4]. *Bidens pilosa* and *Amaranthus* sp. affect rice, corn, and soybean crops; *Conyza* sp. affects mainly soybean, cotton, and corn plantations [5]; and *Echinochloa* sp. is commonly found in rice plantations [6]. These four weeds mentioned have resistance already registered to chemical herbicides [5], especially *Conyza* sp., which is known as one of the most resistant to chemical herbicides [7–9].

Modern agriculture has relied almost exclusively on chemical herbicides, due to their high short-term efficiency and relatively low cost compared to other weed control management technologies [10]. Nonetheless, chemical herbicides present high levels of toxicity and can easily leach into groundwater or enter the food chain. Soil persistence facilitates the development of resistance of some prevalent weed species, continually increasing the demand for the application of these herbicides [11–13]. Accordingly, sustainability-based management strategies, considering an increasing weed resistance scenario, drive the research for other methods to regulate weed infestation [14,15].

Alternatives to the intensive use of chemical herbicides are bioherbicides or bio-based herbicides, which are based on natural living agents, such as bacteria, fungi, plants, and the metabolites of these microorganisms produced during growth [16–20]. Phytopathogens, especially fungi, are among the most studied bioagents, as they have the specific ability to produce significant toxic substances [7,21,22]. The metabolites produced by these microorganisms are capable of penetrating plant leaves, disintegrating their cellular structure, and inducing the production of necrotic lesions or chlorotic halo [13,23].

Submerged fermentation is commonly used in the production of metabolites in studies for bioherbicides [24–26]. Microorganisms are increasingly being studied for this purpose, such as *Pseudomonas aeruginosa*, which, in its cultivation, produces secondary metabolites that inhibit the germination and growth of *Amaranthus hybridus* [27]. In studies with *Fusarium nygamai* chlorosis, necrosis, and inhibition of the root growth of *Striga hermonthica* were obtained [28]. Studies with the application of secondary metabolites of *Fusarium fujikuroi* in *Cucumis sativus* and *Sorghum bicolor* have achieved chlorosis, necrosis, and a decrease in plant height and root [26]. Some strategies are being adopted to maximize the phytotoxic effects on the plants of interest, such as post-fermentation ultrasound-assisted extraction (PF-UAE) [29,30] and co-cultivation techniques [31,32], but they still need to be investigated according to each microorganism.

An ultrasonic field, in the presence of liquids such as water, undergoes the process of acoustic cavitation [33]. This cavitation consists of the formation of low-pressure bubbles that then collapse, generating highly energetic shock waves [34]. Sufficiently energetic shock waves can promote disruption of cell walls and membranes, extracting intracellular material [35]. With the disruption of cell membranes, several metabolites of interest that had not yet been excreted end up being extracted into the medium [36–38]. Furthermore, there is the possibility of extracting molecules from intracellular metabolism, which were not originally present in the fermentation broth. One concern about the use of ultrasound for this application is the possibility of the metabolites of interest being thermal-sensitive, suffering degradation with the thermal energy dissipated by the ultrasound [30].

The idea of joining microorganisms through co-culture, which aims to optimize the production of compounds that are not produced in monoculture and to find new chemical structures, has increased over the years [31,39,40]. Co-cultivation is an approach that involves two or more microorganisms, through interactions between species in the same confined environment, and can be achieved through solid or liquid media [31,41,42]. Cultures interact with each other through cell-to-cell contact or signaling molecules that can lead to the discovery of new chemical substances [42]. Therefore, the existence of different microorganisms in the same medium can affect the morphology of the microorganism, their development, and the synthesis of secondary metabolites [43].

Nevertheless, there are only thirteen bioherbicides registered in the world, nine based on fungi, three on bacteria, and one derived from plant extracts [6,20]. Though the specificity of bioherbicides is an advantageous aspect, it is necessary to shed light on different bioherbicides for each weed of interest [1,44]. Additionally, the development of bio-based products is a recent area of research, and with few commercial products registered worldwide, there is a clear requirement to prospect new bioproducts to contemplate the largest possible number of weeds that affect the current agricultural production [20].

Appropriately, the purpose of this study was to establish a microbial prospection to investigate the potential of viable bioherbicides for the control of *Amaranthus hybridus*, *Bidens*

pilosa, *Conyza* sp., and *Echinochloa crusgalli*, isolating bioagents from several weeds presenting disease symptoms found in areas without the application of herbicides. Moreover, we performed a series of steps to maximize the phytotoxic potential of microorganisms that presented inhibitory activity on these weeds, including the application of the ultrasound-assisted extraction strategy after the fermentation process, microbial co-cultivation, and higher dosages of the fermented broth.

2. Materials and Methods

2.1. Chemicals

Potato Dextrose Agar (PDA) (Sigma-Aldrich), glucose (Dinâmica LTDA[®]), yeast extract (Acumedia[®]), peptone (TM MEDIA), (NH₄)SO₄ (Synth[®]), FeSO₄·7H₂O (Synth[®]), MnSO₄·H₂O (Synth[®]), MgSO₄ (Synth[®]), meat extract (Acumedia[®]), and NaCl (Sigma-Aldrich) were used as purchased without further purification.

2.2. Microbial Prospecting and Isolation of Microorganisms

Pathogenic microorganisms were isolated from weeds that manifested symptoms of disease and lesions possibly caused by some biological agents, located in different municipalities of Rio Grande do Sul, Brazil, in areas without the application of chemical herbicides. Accordingly, Table 1 presents the plant collection locations, the species collected, as well as the geographic coordinates, and the number of microorganisms isolated from each location.

Table 1. Geographic coordinates, plants collected, and the number of microorganisms isolated for this study.

Region	Geographical Coordinates	Collected Species	Number of Isolated Strains
Santa Maria, Brazil	−29.6914 S, −53.8008 W	<i>Conyza</i> sp. And Contaminations *	31
Cerro Largo, Brazil	−28.1506 S, −54.7386 W	<i>Conyza</i> sp. And <i>B. pilosa</i>	6
Marcelino Ramos, Brazil	−27.4673 S, −519085 W	<i>Conyza</i> sp., <i>Spermacoce latifolia</i> , and <i>E. crusgalli</i>	26

* Fungi isolated from contamination of culture plates.

The plant collection was performed from November 2019 to March 2020, and the samples were kept at 4 °C at the Bioprocess Laboratory of the Federal University of Santa Maria, Santa Maria, Brazil, for the isolation of microorganisms. The plants were identified based on morphological characteristics and the separation of each sample was performed with the identification of the plant species and its location. Each injured tissue was transferred to a Petri dish containing PDA culture medium and incubated in a Biochemical Oxygen Demand (B.O.D.) (ELETROLab[®], São Paulo, Brazil) incubator with a photoperiod of 12 h for 7 days at 25 °C [45]. Successive subcultures were performed until a pure culture was obtained. After the isolation of all prospected microorganisms, different culture media were used for microorganisms of fungal or bacterial morphology. To fungi, the subcultures were made with a 6mm disk extracted from the cultured mycelium, and inoculated in a new PDA Petri dish, previously sterilized and prepared [7]. To bacteria, the isolations were performed by a simple streaking technique in a new Petri dish with 1.0 g L^{−1} of meat extract, 5.0 g L^{−1} of peptone, 2.0 g L^{−1} of yeast extract, 5.0 g L^{−1} of NaCl, and 15.0 g L^{−1} of agar, previously sterilized and prepared [46,47].

2.3. Submerged Fermentation

The microorganisms were separated according to their morphology, according to visual characteristics during growth in Petri dishes, and were submitted to different culture media for submerged fermentation. The liquid culture medium for microorganisms with

fungal (LCM-F) morphology consisted of 10.0 g L⁻¹ of glucose, 10.0 g L⁻¹ of yeast extract, 2.0 g L⁻¹ of peptone, 0.5 g L⁻¹ of (NH₄)SO₄, 1.0 g L⁻¹ of FeSO₄·7H₂O, 1.0 g L⁻¹ of MnSO₄·H₂O, and 0.5 g L⁻¹ of MgSO₄, pH 6.0 [26]. The liquid culture medium for the microorganisms that presented bacterial morphology (LCM-B) was prepared with 1.0 g L⁻¹ of meat extract, 5.0 g L⁻¹ of peptone, 2.0 g L⁻¹ of yeast extract, 5.0 g L⁻¹ of NaCl, and pH 7.4 [46]. Inoculation was performed by transferring two 6 mm discs to a 250 mL Erlenmeyer flask containing 125 mL of sterile culture medium. The Erlenmeyer flasks were incubated in an orbital shaker (New Brunswick, model Innova 44) for 7 days for microorganisms with fungal morphology, and 3 days for microorganisms with bacterial morphology, at 28 °C and 120 rpm. After fermentation, the cells were filtered (Whatman®, Merck KGaA, Darmstadt, Germany) and separated by centrifugation at 4000 rpm for 20 min (Eppendorf, model 5804R), and the supernatant was subjected to membrane filtration (Merck Millipore TM), with a pore size of 0.45 µm and diameter of 47 mm [7]. The filtered supernatant was used in the bioassays. The supernatant was incubated to verify microbial growth, with negative results. The 63 microorganisms were considered distinct treatments for the tests, and the bioherbicidal activity was evaluated by a series of analyses.

2.4. Strategies to Increase the Herbicidal Activity of Fermentation Broth

2.4.1. Co-Cultivation Medium

The selection of microorganisms was performed initially by 10 combinations of pairs of microorganisms cultivated in Petri dishes to evaluate the viability of co-cultivation, and to verify possible unwanted inhibitions between strains. Each combination was composed of two microorganisms, and the number of combinations was determined through combinatorial analysis for simple combinations. The combinations were performed with the inoculation of a 6 mm disk extracted from the Petri dish culture of each microorganism in a new PDA Petri dish, previously sterilized and prepared. After selecting the best combinations, fermentation was carried out, and a specific culture medium was used for each morphology. For combinations between microorganisms that presented fungal morphology, the LCM-F was utilized [26]. Considering the combinations of microorganisms with bacterial morphology, the LCM-B was utilized [46]. Finally, for the combinations with fungi and bacteria, the addition of all the components of both media (LCM-F plus LCM-B) was performed [26,46]. The culture media were incubated and maintained under conditions as described above.

2.4.2. Post-Fermentation Ultrasound-Assisted Extraction (PF-UAE)

After 7 days of fermentation, the broth and biomass were directly subjected to Ultrasound-Assisted Extraction (UAE). An ultrasonic processor, UP400S (Hielscher Ultrasonics GmbH), Sonotrode H22 (Hielscher Ultrasonics GmbH) with a diameter of 22 mm was used. Sonication was performed directly by coupling a sonication cell with a cooling bath at 10 °C, and inside the cell, it was filled with the fermentation broth with the biomass. The process conditions were 90% amplitude and 0.5 s cycle for 40 min [48]. Afterward, the cells were separated from the medium, centrifuged at 4000 rpm for 20 min (Eppendorf, model 5804R), and filtered by membrane (Merck Millipore TM) with a pore size of 0.45 µm and a diameter of 47 mm to obtain a cell-free supernatant [7].

2.4.3. Co-Cultivation with PF-UAE

Assays were performed associating co-cultivation with the use of PF-UAE. The co-cultivation was conducted as described in Section 2.4.1, and after the fermentation period, the broth and biomass were submitted to UAE as described in Section 2.4.2.

2.5. Application in Plant Species

2.5.1. Bioherbicidal Activity in *C. sativus*

The 63 microorganisms isolated were considered distinct treatments, and each treatment consisted of four plants of *C. sativus*, and each plant was considered a repetition

(experimental unit). Sowing was performed in trays; after emergence, the seedlings were replanted in 1 L pots containing 50% soil and 50% Mecplant[®] commercial substrate. The seeds were obtained from the local market, without any pre-sowing treatment. In this first stage, each application was performed with a manual sprayer, with the application of 1 mL per plant, directly on the shoot. For the application in the control plants, the broth was replaced with distilled water. The application was performed 9 days after sowing. The visual assessment of phytotoxicity was performed 21 days after application (DAA), based on the scale of Frans and Crowley (1986) [49].

2.5.2. Bioherbicidal Activity on Weeds

The best results obtained in the assays with *C. sativus* were selected, and the bioherbicidal activity in *Amaranthus* sp., *Conyza* sp., *B. pilosa*, and *E. crusgalli* were evaluated. The plants were sown and replanted into 1 L pots containing commercial Mecplant[®] substrate and kept in a greenhouse. Applications were made with a manual sprayer.

To evaluate the bioherbicidal activity of the crude broth, 1 mL of supernatant per plant was applied, in 4 repetitions, where each plant was considered a repetition. For the control plants, the broth was replaced with distilled water [50]. The crude broth assay and control plants were kept under the same conditions, and the evaluations were performed at 21 DAA. The evaluation assays of the processes with PF-UAE, co-cultivation, and a triple application of broth, consisted only of the application in *A. hybridus* and *E. crusgalli*. One seedling of *A. hybridus* and 2 to 3 seedlings of *E. crusgalli* were replanted in pots; each pot was considered a plant, and each plant a repetition. For one of the methodologies, the triple crude broth was applied; that is, 3 mL per plant, and the others followed as previously described [25]. The applications were conducted in 7 repetitions, and the evaluations were carried out in 14 DAA. In all weed applications, plant height (PH), root length (RL), shoot fresh mass (SFM), root fresh mass (RFM), shoot dry mass (SDM), and root dry mass (RDM) were evaluated [51].

2.6. Identification of Microorganisms

The molecular identification of promising microorganisms was performed by the Biological Institute of São Paulo, São Paulo, Brazil. The DNA of the isolates was extracted according to the method described by [52] from the microorganism grown in a culture medium. The extracted genomic DNA sample was subjected to a polymerase chain reaction (PCR) for amplification of the ITS (internal transcribed spacer) region of the rDNA (isolates A14.2 and B22.2), beta-tubulin gene segment (γ isolate), and *gyrB* gene segment (B14 isolate). The oligonucleotide primers for the ITS region were SR6R (5'-AAGWAAAAGTCGTAACAAGG-3') and LR1 (5'-GGTTGGTTTCTTTTCCT-3') (Vilgalys and Hester, 1990); for the beta-tubulin gene, they were TUB2Fd (5'-GTBCACCTYCARACC GGYCARTG-3') and TUB4Rd (5'-CCRGAYTGRCCRAARACRAAGTTGTC-3') [53]; and for the *gyrB* gene, they were *gyrB*-F (5'-GTNYAYCGTGAYGGNAAAATYCA-3') and *gyrB*-R (5'-GCAGARTCWCCCTCTACRATATA-3') (developed by R. Harakava, Biological Institute of São Paulo). The PCR mix consisted of 1 μ L of DNA, 1 μ L of each primer at 10 μ M, 10 μ L of 5X PCR buffer, 1 μ L of 10 mM dNTPs, 0.2 μ L of GoTaq DNA polymerase 5U/ μ L (Promega), and 35.8 μ L autoclaved MilliQ H₂O, to a final volume of 50 μ L. The amplification program consisted of initial denaturation at 94 °C/2 min followed by 40 cycles of denaturation at 94 °C/10 s, annealing at 54 °C/30 s, extension at 72 °C/45 s, and final extension at 72 °C/4 min. The verification of the amplified products was performed by electrophoresis in a 0.8% agarose gel stained with ethidium bromide. The amplified products were purified by precipitation with polyethylene glycol, directed to sequencing reaction by the chain termination method using the Big Dye 3.1 reagent (Applied Biosystems), and analyzed in a 3500 xL automatic capillary sequencer (Applied Biosystems) [54]. For the construction of the phylogenetic trees, the Neighbor-Joining method was used with a bootstrap of 1000 repetitions in the MEGA 6.0 program [55].

2.7. Statistical Performance

The means were compared by Student's *t*-test in the Statistica® software, version 8.0 [56], using a probability of error lower than 5% ($p \leq 0.05$) as a significant difference criterion.

3. Results

3.1. Initial Bioprospecting

Considering the 63 different strains of microorganisms isolated, 21 microbial strains exhibited visual phytotoxic effects on *C. sativus* plants (Figure 1), such as necrosis, yellowing, and leaf spots. The others showed no visual effects on the plants. The effects were already observed in the first week of evaluation.

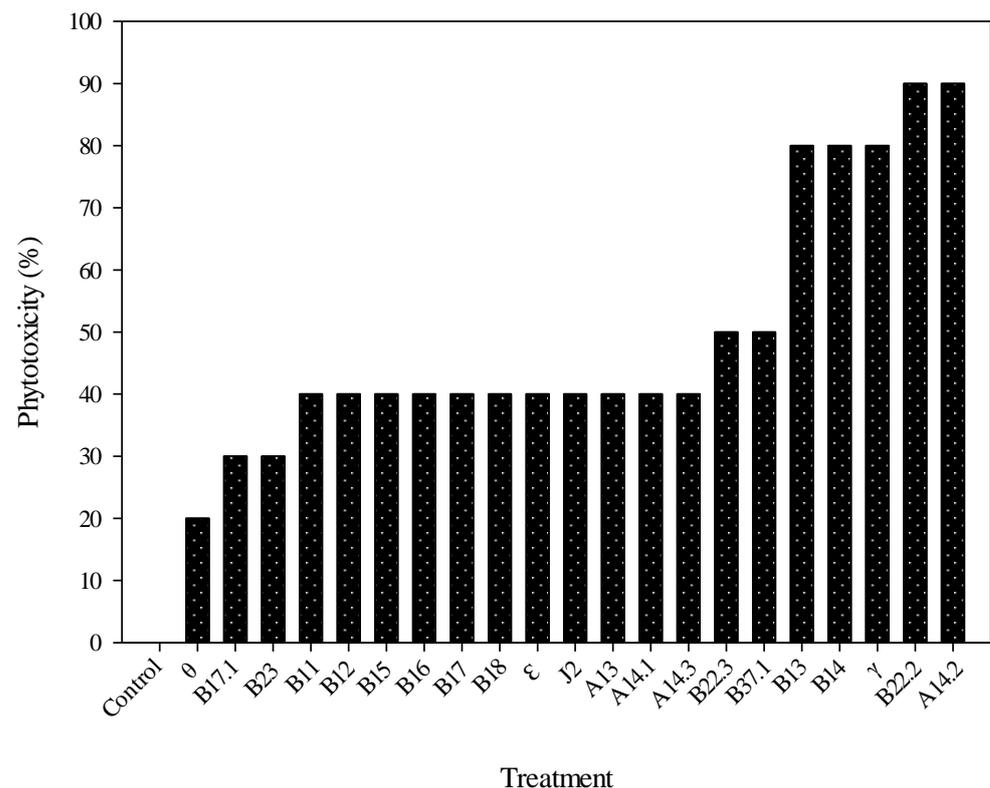


Figure 1. Phytotoxicity (%) of supernatants at 21 days after application (DAA) in *C. sativus*.

Five microorganisms presented significant results concerning phytotoxicity: strain B22.2, strain γ , strain A14.2, strain B13, and strain B14, as they presented mortality of at least one of the plants in the stand, and phytotoxicity equal to or higher than 80%. The other microorganisms showed less intense effects and plant recovery throughout the trial evaluation period.

3.2. Inhibition Potential on the Studied Weeds

Table 2 presents the quantitative evaluation of six different variables studied, namely: plant height (PH), root length (RL), shoot fresh mass (SFM), root fresh mass (RFM), shoot dry mass (SDM), and root dry mass (RDM).

Table 2. Phytotoxic effects of microorganism metabolites on *Conyza* sp., *B. pilosa*, *A. hybridus*, and *E. crusgalli* plants after 21 days after application (DAA).

	PH (cm)	RL (cm)	SFM (g)	SDM (g)	RFM (g)	RDM (g)
<i>E. crusgalli</i>						
B13	37.0 ± 5.4	32.5 ± 2.5 ^G	12.482 ± 3.787 ^I	2.572 ± 0.118 ^I	7.275 ± 2.443	3.316 ± 0.972
B14	31.0 ± 1.4 ^I	29.0 ± 7.0	8.462 ± 3.684 ^I	1.931 ± 0.885 ^I	5.060 ± 2.101 ^I	2.261 ± 0.854 ^I
A14.2	31.0 ± 4.3 ^I	28.9 ± 7.8	12.151 ± 3.546 ^I	3.524 ± 1.030	7.862 ± 3.759	3.207 ± 1.170
B22.2	37.5 ± 3.5	25.4 ± 4.3	9.370 ± 2.088 ^I	2.589 ± 0.353 ^I	6.733 ± 2.293	3.119 ± 0.669
γ	34.1 ± 1.4 ^I	24.0 ± 1.8	16.871 ± 4.150 ^I	3.880 ± 0.877	15.662 ± 9.624	4.801 ± 0.847
Control	38.3 ± 2.3	24.0 ± 1.8	25.733 ± 1.872	4.700 ± 0.529	10.633 ± 2.669	4.200 ± 0.557
<i>A. hybridus</i>						
B13	18.0 ± 5.6 ^I	17.5 ± 2.1	2.465 ± 1.085 ^I	0.902 ± 0.386 ^I	1.174 ± 0.734	0.607 ± 0.390
B14	29.5 ± 1.7	22.0 ± 2.4	5.233 ± 1.062 ^I	2.542 ± 0.539	2.490 ± 0.662	1.680 ± 0.355
A14.2	24.8 ± 0.5 ^I	18.3 ± 2.6	7.254 ± 0.528	2.221 ± 0.164 ^I	1.497 ± 0.397	0.545 ± 0.169
B22.2	21.9 ± 11.5	18.1 ± 2.7	4.720 ± 3.684	1.460 ± 1.194 ^I	1.928 ± 1.211	0.854 ± 0.525
γ	24.9 ± 8.1	17.3 ± 6.8	3.987 ± 2.894 ^I	1.318 ± 0.953 ^I	1.470 ± 1.171	0.697 ± 0.580
Control	31.0 ± 1.5	22.5 ± 5.8	8.355 ± 1.062	3.337 ± 0.043	2.450 ± 1.343	1.247 ± 0.769
<i>B. pilosa</i>						
B13	22.9 ± 2.4	27.3 ± 4.1	6.478 ± 2.115	1.855 ± 0.425	1.431 ± 0.460	0.875 ± 0.037 ^G
B14	22.0 ± 2.9	28.8 ± 3.0	4.859 ± 1.745	1.632 ± 0.796	1.112 ± 0.405	0.793 ± 0.144
A14.2	23.4 ± 2.0	29.0 ± 0.0	4.137 ± 0.867	1.781 ± 0.394	0.964 ± 0.273	0.763 ± 0.198
B22.2	16.5 ± 5.1 ^I	26.3 ± 5.6	2.572 ± 1.441	0.854 ± 0.562	0.660 ± 0.474	0.389 ± 0.268
γ	20.5 ± 1.3 ^I	14.0 ± 1.0	4.347 ± 2.081	1.213 ± 0.695	1.004 ± 0.628	0.438 ± 0.262
Control	24.6 ± 1.9	25.5 ± 10.7	4.460 ± 1.886	1.553 ± 0.374	1.089 ± 0.581	0.520 ± 0.184
<i>Conyza</i> sp.						
B13	21.0 ± 2.2	18.3 ± 2.5	9.695 ± 3.687	2.368 ± 0.545	8.285 ± 2.280	1.900 ± 0.466 ^G
B14	22.5 ± 3.0	23.5 ± 7.7	12.175 ± 4.413	2.515 ± 0.821	12.797 ± 0.476 ^G	2.315 ± 1.007 ^G
A14.2	18.0 ± 5.0	18.5 ± 5.2	6.290 ± 3.038	1.515 ± 0.769	6.538 ± 2.354	1.510 ± 0.653
B22.2	25.5 ± 5.2	24.0 ± 5.8	13.493 ± 6.620	2.710 ± 1.369	12.138 ± 6.080	2.265 ± 1.589
γ	19.3 ± 5.1	14.8 ± 4.5	10.775 ± 6.366	2.360 ± 1.329	8.238 ± 4.465	1.735 ± 1.052
Control	24.5 ± 4.7	19.0 ± 0.8	10.585 ± 4.826	2.010 ± 1.066	6.263 ± 2.333	0.850 ± 0.417

^I Significant inhibition according to Student's *t*-test, 95% confidence. ^G Significant growth according to Student's *t*-test, 95% confidence.

In plants of *Conyza* sp., no significant inhibitory effects were observed. Similar results were found for the species, *B. pilosa*, which indicated a reduction in plant height only in A14.2 (33%) and in B22.2 (16%). The other variables showed no inhibition effects. The *E. crusgalli* and *A. hybridus* plants presented higher sensitivity to the treatments applied. In *E. crusgalli* plants, all treatments indicated inhibitory effects of fresh and dry mass, both in shoots and in roots. The treatment, B14, showed the best inhibition rates in PH (19%), and had variations between 52 and 67% of inhibition in the variables, SFM, RFM, SDM, and RDM. The results observed in *A. hybridus* plants demonstrated the ability of the B13 treatment to reduce PH, SFM, and SDM, with reduction percentages between 22 and 73%. Additionally, no visible phytotoxicity lesions were observed in any of the treatments and plants.

3.3. Strategies to Increase the Herbicidal Activity of the Fermented Broth

Considering the results obtained in the tests of application of the crude broth, the two species that suffered significant effects (*E. crusgalli* and *A. hybridus*) were selected. Tests were performed with different methods after fermentation to potentiate the inhibitory effects observed in the previous stage of the study. Tables 3 and 4 indicate the results of all the methods proposed and applied to *E. crusgalli* and *A. hybridus* plants.

Table 3. Effects of metabolites from the crude broth, triple dose and UAE, co-cultivation, and co-cultivation and PF-UAE on *E. crusgalli* parameters at 14 days after application (DAA).

	PH (cm)	RL (cm)	SFM (g)	SDM (g)	RFM (g)	RDM (g)
Raw broth—Triple dose						
B13	53.9 ± 3.1	29.3 ± 3.6 ^I	17.097 ± 4.014 ^I	1.698 ± 0.313 ^I	3.010 ± 0.901	0.305 ± 0.043 ^I
B14	55.6 ± 4.2	27.7 ± 1.1 ^I	16.218 ± 4.909 ^I	1.734 ± 0.380 ^I	2.012 ± 0.536 ^I	0.251 ± 0.079 ^I
B22.2	56.4 ± 3.2	31.3 ± 3.8	16.440 ± 3.366 ^I	2.013 ± 0.278 ^I	2.301 ± 0.318	0.435 ± 0.081
γ	55.0 ± 4.8	22.7 ± 2.4 ^I	9.743 ± 2.005 ^I	1.210 ± 0.378 ^I	0.539 ± 0.176 ^I	0.127 ± 0.052 ^I
A14.2	55.4 ± 2.7	32.9 ± 2.6	22.639 ± 2.683	2.286 ± 0.308	3.801 ± 0.859 ^G	0.457 ± 0.092
Raw broth—UAE						
B13	61.4 ± 4.8	30.7 ± 4.4	22.394 ± 2.610	1.807 ± 0.235 ^I	2.416 ± 0.344	0.247 ± 0.034 ^I
B14	55.9 ± 2.3	28.0 ± 2.5 ^I	16.360 ± 5.431 ^I	1.718 ± 0.441 ^I	1.744 ± 0.665 ^I	0.312 ± 0.126 ^I
B22.2	49.0 ± 3.9 ^I	28.4 ± 2.2 ^I	9.002 ± 2.591 ^I	1.295 ± 0.189 ^I	1.478 ± 0.513 ^I	0.231 ± 0.055 ^I
γ	61.6 ± 4.2	31.1 ± 4.3	22.933 ± 5.726	2.135 ± 0.549	2.683 ± 0.621	0.296 ± 0.088 ^I
A14.2	47.7 ± 1.9 ^I	26.9 ± 2.7 ^I	12.095 ± 1.870 ^I	0.985 ± 0.245 ^I	1.789 ± 0.266 ^I	0.198 ± 0.079 ^I
Co-cultivation						
A14.2/B22.2	53.4 ± 2.9	30.4 ± 4.4	15.171 ± 2.802 ^I	1.702 ± 0.293 ^I	1.414 ± 0.475 ^I	0.272 ± 0.091 ^I
B13/B14	55.1 ± 3.0	34.4 ± 3.4	20.064 ± 4.061	2.027 ± 0.505	2.296 ± 0.566	0.371 ± 0.129
B14/γ	63.3 ± 3.9 ^G	30.3 ± 4.0	25.727 ± 3.922	2.227 ± 0.290	2.941 ± 0.502	0.349 ± 0.073
B22.2/γ	57.7 ± 3.6	31.1 ± 3.4	23.787 ± 3.934	2.375 ± 0.371	3.223 ± 0.641	0.413 ± 0.111
Co-cultivation and UAE						
A14.2/B22.2	57.1 ± 4.4	29.6 ± 3.8 ^I	12.992 ± 2.178 ^I	1.438 ± 0.200 ^I	1.426 ± 0.388 ^I	0.194 ± 0.055 ^I
B13/B14	59.4 ± 3.4	32.9 ± 3.2	19.930 ± 5.065	2.038 ± 0.356 ^I	2.430 ± 0.575	0.283 ± 0.068 ^I
B14/γ	56.9 ± 3.6	27.1 ± 1.9 ^I	15.890 ± 3.856 ^I	1.697 ± 0.329 ^I	0.917 ± 0.167 ^I	0.203 ± 0.067 ^I
B22.2/γ	59.9 ± 1.8	30.3 ± 1.8 ^I	18.420 ± 4.003 ^I	1.828 ± 0.407 ^I	1.818 ± 0.640 ^I	0.260 ± 0.112 ^I
Control	57.4 ± 4.5	33.7 ± 2.9	22.298 ± 1.982	2.434 ± 0.228	2.769 ± 0.529	0.437 ± 0.081

^I Significant inhibition according to Student's *t*-test, 95% confidence. ^G Significant growth according to Student's *t*-test, 95% confidence.

Table 4. Effects of metabolites from the crude broth, triple dose, and PF-UAE, as well as co-cultivation, and co-cultivation with PF-UAE on *A. hybridus* parameters at 14 days after application (DAA).

	PH (cm)	RL (cm)	SFM (g)	SDM (g)	RFM (g)	RDM (g)
Raw broth—Triple dose						
B13	19.0 ± 3.0	32.1 ± 5.6	3.832 ± 1.284 ^G	0.573 ± 0.136 ^G	0.664 ± 0.237 ^G	0.100 ± 0.039 ^G
B14	16.2 ± 2.0 ^I	36.0 ± 6.9 ^G	3.023 ± 0.698	0.476 ± 0.088	0.502 ± 0.149 ^G	0.081 ± 0.027
B22.2	19.0 ± 2.2	39.4 ± 5.7 ^G	3.772 ± 0.888 ^G	0.603 ± 0.129 ^G	0.511 ± 0.177 ^G	0.108 ± 0.036 ^G
γ	16.0 ± 2.4 ^I	25.9 ± 7.5	2.466 ± 1.100	0.464 ± 0.116	0.312 ± 0.137	0.060 ± 0.027
A14.2	23.0 ± 3.8	45.0 ± 8.2 ^G	5.660 ± 1.396 ^G	0.800 ± 0.104 ^G	0.946 ± 0.302 ^G	0.161 ± 0.036 ^G
Raw broth—UAE						
B13	21.0 ± 2.8	30.4 ± 2.4	3.567 ± 0.833 ^G	0.446 ± 0.201	0.516 ± 0.133 ^G	0.091 ± 0.024 ^G
B14	18.4 ± 2.3	35.1 ± 9.4	2.734 ± 0.631	0.457 ± 0.082	0.292 ± 0.055	0.061 ± 0.021
B22.2	18.6 ± 4.0	30.1 ± 6.0	3.367 ± 1.121	0.538 ± 0.124 ^G	0.548 ± 0.228 ^G	0.079 ± 0.040
γ	21.1 ± 2.3	18.1 ± 5.1 ^I	0.843 ± 0.273 ^I	0.198 ± 0.035 ^I	0.083 ± 0.045 ^I	0.018 ± 0.009 ^I
A14.2	11.6 ± 2.4 ^I	36.4 ± 6.9 ^G	4.571 ± 1.439 ^G	0.694 ± 0.173 ^G	0.788 ± 0.286 ^G	0.132 ± 0.048 ^G
Co-cultivation						
A14.2/B22.2	23.4 ± 3.4	24.9 ± 7.9	3.384 ± 1.024	0.468 ± 0.114	0.440 ± 0.263	0.065 ± 0.015
B13/B14	19.0 ± 1.7	25.3 ± 4.3	2.662 ± 0.506	0.413 ± 0.061	0.312 ± 0.111	0.055 ± 0.021
B14/γ	16.3 ± 2.3 ^I	20.1 ± 3.3 ^I	1.420 ± 0.394 ^I	0.271 ± 0.040 ^I	0.129 ± 0.068 ^I	0.031 ± 0.015 ^I
B22.2/γ	17.4 ± 2.6	25.0 ± 2.9	2.139 ± 0.441	0.398 ± 0.042	0.226 ± 0.054 ^I	0.038 ± 0.008 ^I
Co-cultivation and UAE						
A14.2/B22.2	22.7 ± 5.6	37.0 ± 6.4 ^G	4.972 ± 1.655 ^G	0.679 ± 0.202 ^G	0.896 ± 0.255 ^G	0.138 ± 0.045 ^G
B13/B14	16.6 ± 4.1	27.4 ± 11.5	2.134 ± 1.000	0.425 ± 0.145	0.273 ± 0.059	0.058 ± 0.027
B14/γ	19.8 ± 2.6	31.1 ± 3.0	3.367 ± 0.651 ^G	0.614 ± 0.152 ^G	0.479 ± 0.141 ^G	0.091 ± 0.025 ^G
B22.2/γ	18.5 ± 3.9	27.3 ± 1.9	2.703 ± 0.709	0.457 ± 0.072	0.312 ± 0.086	0.050 ± 0.013
Control	20.2 ± 2.3	27.3 ± 5.2	2.567 ± 0.504	0.414 ± 0.053	0.311 ± 0.076	0.058 ± 0.011

^I Significant inhibition according to Student's *t*-test, 95% confidence. ^G Significant growth according to Student's *t*-test, 95% confidence.

3.4. Triple Dose Application

In *E. crusgalli* plants (Table 4), the best treatments with the application of triple dosage were B14 and γ , which only did not change the PH. B14 remained with the same amount of inhibition as presented in the crude broth with a single dose. The treatments, B13 and B14, indicated some wilting symptoms in *E. crusgalli* plants after 3 DAA, indicating possible effects. Nevertheless, the plants recovered until the finalization of the experimental step.

Moreover, the treatments with B13 and γ demonstrated that the application of more doses influences the inhibition, doubling the number of inhibited variables. The opposite was observed in the treatment with B22.2, where better results were not observed with a higher volume of broth applied.

3.5. Post-Fermentation Ultrasound-Assisted Extraction (PF-UAE) of the Raw Broth

The treatments of strains, B13, B14, and B22.2, did not show significant inhibition effects on *A. hybridus* plants. The results were better with crude broth application than when compared with the PF-UAE.

The best effect on *A. hybridus* was when the γ treatment was applied, which indicated significant results in RL (33%), SFM (67%), SDM (52%), RFM (73%), and RDM (70%). Compared with the application of the broth without the PF-UAE, an improvement in the bioherbicidal activity was observed, evidencing the potential of this microorganism.

Inhibitions were higher in *E. crusgalli* plants, where the treatments, B22.2 and A14.2, presented the best inhibitory effects, influencing the development of all variables. Inhibitions ranged from 15% to 60% for B22.2, and 17% to 60% for A14.2. Compared with the application of the raw broth, it was observed that the PF-UAE improved the efficiency of most treatments applied to *E. crusgalli*.

3.6. Co-Cultivation Medium

The tests were performed with the addition of microorganisms from the row and column from the matrix presented in Table 5. The data are expressed as the first symbol referring to the row and the second referring to the column. The – sign indicates an inhibition effect and a + indicates a growth effect of the microorganism.

Table 5. Matrix of inhibition and growth results of the 10 combinations of microorganisms in a Petri dish.

Code	γ	B22.2	B14	B13	A14.2
A14.2	– +	++	– +	– +	
B13	– +	+ –	++		
B14	++	+ –			
B22.2	++				
γ					

The selected combinations were A14.2/B22.2, B13/B14, B14/ γ , and B22.2/ γ . In this methodology, the broth of the co-cultivation of the microorganisms, B14 and γ , presented better performance than the other treatments in the application in plants of *A. hybridus*, besides obtaining better performance than those evaluated alone.

The treatment A14.2/B22.2 had no effects on *A. hybridus*, but the opposite was observed in *E. crusgalli* plants, where this treatment was the only one that presented the inhibition of variables. The inhibitions observed were in the fresh and dry mass, both in the shoots and roots.

3.7. Post-Fermentation Ultrasound-Assisted Extraction (PF-UAE) of the Co-Cultivation Broth

Evaluating the plants of *E. crusgalli* with the treatment derived from PF-UAE over co-cultivation, a significant improvement was observed against the treatment of co-cultivation broth without UAE, also compared to the raw broth. In *E. crusgalli*, A14.2/B22.2 presented a

higher percentage of reduction of variables compared to the treatment of co-culture without UAE, also showing a reduction of CR (12%). Metabolites from the co-cultures, B14/ γ and B22.2/ γ , showed inhibition of five variables in *E. crusgalli* both with and without the use of PF-UAE over the broth.

In *A. hybridus*, growth effects are systematically observed in all methodologies explored, with suspicion of troubles in the growth of the control plants, since the masses of the control plants are systematically lower than the average masses of the other tested plants. It is important to note the positive results achieved in the first assays carried with raw broth (Table 2).

3.8. Microbial Identification

The morphology of the microorganisms was visually determined according to the growth in the Petri dish. Strain B14, showed bacterial morphology, whereas the strains, A14.2, B22.2, and γ , showed fungal morphology. The treatments, B14/ γ , and strains, A14.2 and B22.2, from UAE inhibited all variables in the methodologies previously tested; therefore, the four strains that compose these treatments were selected for identification (A14.2, B14, B22.2, and γ). Strain B14 was identified as *Bacillus velezensis*—GenBank DQ903176, strain γ as *Aspergillus flavus*—GenBank AY017536, and strains A14.2 and B22.2 belong to the same species: *Nigrospora sphaerica*—GenBank KX985964.

4. Discussion

4.1. Initial Bioprospecting

The results presented in the applications of *C. sativus* indicated that the broths from these microorganisms are promising, and the bioherbicide potential in weeds can be evaluated. The other microorganisms manifested less intense effects and recovery of the plants throughout the trial evaluation period.

Accordingly, the initial application in *C. sativus* gives considerable information to the next steps. *C. sativus* is commonly explored to perform bioassays, since it is a plant with high sensitivity to phytotoxic compounds, and is easy to grow [26,57]. Yellowing and leaf spots were also reported in studies, which led to the selection and identification of the fungus, *Diaporthe* sp. [57]. The fungus, *Fusarium fujikuroi*, was also selected by prospecting for microorganisms tested in *C. sativus*, showing dark spots and leaf chlorosis [26].

4.2. Inhibition Potential on the Studied Weeds

The four weeds initially evaluated have an important commercial interest, since they are known for affecting the production of important crops. *Conyza* sp. affects the crops of soy, cotton, and corn [5,58]; *B. pilosa* and *A. hybridus* affect the crops of rice, corn, and soy [4,5]; and *E. crusgalli* affects mainly rice crops [5,59]. Moreover, all these four weeds have been reported to exhibit resistance to commercial herbicides [5].

In these weeds, the six evaluated variables (PH, RL, SFM, RFM, SDM, RDM) were chosen for being meaningful in the impairing potential of the weeds over the crops. The reduction in weed height decreases the intensity of available light, due to the shading caused by the crop of interest, affecting its photosynthesis. Therefore, the reduction in root length/mass reduces the consumption of nutrients, such as water absorption, nitrogen compounds, and minerals, reducing weed development, as well as increasing the availability of these nutrients for the crop of interest [60–62].

Promissory results were observed in *E. crusgalli* and *A. hybridus*. Some reports in the literature already present studies of microorganisms, such as *Lasiodiplodia pseudotheobromae*, *Pseudomonas aeruginosa*, and *Carum carvi*, promissory for these species [27,63]. Studies have reported a reduction in height and shoot and root fresh and dry mass of *E. crusgalli* and *B. pilosa* from metabolites of the fungus, *Diaphorte schini* [51]. Furthermore, few studies present the biological behavior of the plant when submitted to secondary metabolite phytotoxins [20]. The reduction of weed fresh mass was also observed with the application of the secondary metabolites of *Phoma dimorpha* in *Senna obtusifolia* plants [64]. The delay

and/or inhibition of plant growth may be associated with phytotoxin interference in the biosynthesis of amino acids, such as valine, leucine, and isoleucine, which are essential for plant growth and better performance [51,65].

The unsatisfactory results for *Conyza* sp. may be related to the fact that the genus, *Conyza*, has records of resistance to several chemical herbicides, such as Paraquat, glyphosate, and 2,4-dichlorophenoxyacetic acid (2,4-D) [5], besides being known to be one of the most resistant to chemical herbicides [7–9]. The species, *B. pilosa*, also gives insufficient results, which only presented a significant reduction in plant height in A14.2 (33%) and B22.2 (16%). The other variables showed no inhibition effects. The low effects may be associated with the low concentration of metabolites in the supernatant [66].

Due to the lack of significant results from the application tests of the crude broth on the *Conyza* sp. and *B. pilosa*, the next steps were carried out with the two species that were susceptible to the treatments (*E. crusgalli* and *A. hybridus*).

4.3. Strategies to Increase the Herbicidal Activity of the Fermented Broth

The low concentration of metabolites may be associated with the lack of phytotoxic results, as well as the rapid volatilization of the product, causing the broth not to be absorbed by plant tissues [25,67–69]. Moreover, some bioherbicides of microbiological origin require post-fermentation steps, as well as the use of adjuvants, in search of methods that improve product efficiency [50,68]. Aiming to potentialize the inhibitory effects over these weeds, tests were performed with four different methods: dose augmentation, co-cultivation of microorganisms, post-fermentation UAE (PF-UAE) of raw broth, and PF-UAE over co-cultivation broth. Higher dosage aims to directly increase the amounts of metabolites available to interact with the plant tissues, whereas co-cultivation and PF-UAE can give rise to synergetic effects between metabolites, or even the emergence of new ones.

4.4. Triple Dose Application

The increase in the dose of broth applied aims to simulate the equivalent effect of the application of broths with a higher concentration of metabolites, and it is commonly used in agriculture, as it aims to increase the effectiveness of the applied product [70]. Wilt symptoms were also observed with the application of secondary metabolites produced by *Phoma* sp. [25]. The doubling in the number of inhibited variables for treatments with B13 and γ with the increase of application dose indicates that these microorganisms have the potential for bioherbicidal activity in *E. crusgalli*. Studies indicated that increasing the applied dose potentiates the expected effects on plants, as it consequently increases the number of metabolites disposed of in the plant [25,71].

4.5. Post-Fermentation Ultrasound-Assisted Extraction (PF-UAE) of the Raw Broth

The worst results obtained with the application of PF-UAE in bacteria (strains B13 and B14) may be associated with the metabolites being sensitive to the effects of the process. Bacteria have a few complex structures, such as protein compartments that have some enzymes necessary for cellular metabolism, and there are not many intracellular products to be extracted [72,73]. Compared to the application of the raw broth, it was observed that the PF-UAE improved the efficiency of most treatments applied to *E. crusgalli*. Some metabolites can injure and damage some weed species more than others [74]. The γ treatment showed significant results in *A. hybridus* and not in *E. crusgalli*. The application of this methodology indicated that the possible metabolites from the intracellular medium have more pronounced effects on grasses. The more pronounced improvements were in microorganisms with fungal morphology, indicating that the higher cellular complexity of the fungi possibly plays a role in the achieved results. Fungal growth can be filamentous, with dispersed hyphae, or in the form of spherical pellets, consisting of compact hyphae [75,76]. These microorganisms grew in the form of pellets, which may interfere with the transport of substrates into the cell, which may have significant effects on cellular metabolism and the synthesis of products and defense mechanisms [77].

As fungi are eukaryotic organisms, the complexity of the intracellular environment is higher when compared to the prokaryotic cells of bacteria [77]. Fungi have a range of organelles, including fungal vacuoles that are responsible for producing and storing digestive enzymes, among other functions [78,79]. Considering these differences between fungal and bacterial cells, the PF-UAE over the intracellular components from fungi tends to be more promising compared to bacteria, given the higher diversity of metabolites, mainly digestive enzymes found in fungal cells [79].

4.6. Co-Cultivation Medium

This technique promotes advantages in improving quality; it has potential in the replacement of substrates, but mainly stands out for the increase in product yield [80]. Different metabolites may have been produced by co-cultivation, which improved the performance of the treatment with the γ microorganism, and reinforced the bioherbicidal potential of the B14 microorganism, which had already shown efficiency in other methodologies. This is due to competition between microorganisms, which, when inoculated together, are exposed to stress-inducing factors that stimulate gene complexes that have not yet been expressed under normal culture conditions and can induce the production of new compounds [32,81]. For the other treatments, co-cultivation was not more efficient than the crude broth in *A. hybridus*. Additionally, to the production of new compounds, the loss of some metabolites present in the culture may also occur when submitted to co-cultivation [81].

4.7. Post-Fermentation Ultrasound-Assisted Extraction (PF-UAE) of the Co-Cultivation Broth

The combination of PF-UAE and co-cultivation techniques promotes a few significant improvements compared with these two techniques employed isolated, mainly in A14.2/B22.2 cultivation over *E. crusgalli*. This is possibly due to the synergy in the characteristics of these two techniques, as already discussed in Sections 4.5 and 4.6.

Studies claimed that *Amaranthus* is among the most difficult species to control globally, and in field experiments, *A. hybridus* was indicated to be more resistant to treatment applications than *E. crusgalli* [82].

Growth effects are systematically observed in all methodologies explored (PF-UAE and co-cultivation medium) against *A. hybridus*, where it was observed that the averages of all parameters of the control were lower than most treatments. This observation may be directly related to the inadequate development of the control plants, as some factors influence plant evolution, such as water, temperature, and light, which is the most important variable that affects photosynthesis and plant growth and development [65]. The hypothesis of problems with the growth of control is reinforced by the positive results achieved in the first assays carried with raw broth, presented in Sections 3.2 and 4.2.

4.8. Microbial Identification

The properties and applications of *Bacillus velezensis* have been studied both in agriculture and in the fermentation industry. This microorganism is easy to grow and has a high ability to resist stress. The literature presents reports of applications as a pesticide to combat classified airborne fungi or soil diseases [83]. Furthermore, some reports indicated *Nigrospora sphaerica* as an important species against the water hyacinth [84]. Additionally, in Table 4, the results show *A. hybridus* presents higher resistance compared to *E. crusgalli* plants. The significant differences mostly refer to growth. There was only inhibition in the plant height with B14 and γ treatment. Growth effects are systematically observed in all methodologies used (UAE and co-cultivation medium) against *A. hybridus*, where it was verified that the averages of all parameters of the controls were lower than most treatments. The microorganisms presented by the bioprospecting performed indicated a high potential to produce secondary metabolites for weed control.

5. Conclusions

The purpose addressed in this study was promising in obtaining a bioherbicide for weed control through the production of metabolites by submerged fermentation. The highest growth-inhibiting effects on *E. crusgalli* plants were obtained when the A14.2 and B22.2 (*N. sphaerica*) broth with biomass was submitted to PF-UAE for cell disruption. The metabolites from the co-cultivation of B14 (*B. velezensis*) with γ (*A. flavus*) were indicated to be significantly efficient in inhibiting the development of *A. hybridus*. The exploration of methodologies such as co-cultivation and ultrasound-assisted extraction proved to be excellent tools for obtaining new compounds, which may be promising in the production of a bioherbicide. The results obtained encourage the development of more studies of concentration and formulation for the potentiation of the bioherbicidal activity of the metabolites coming from the microorganisms explored in the bioprospecting performed in this study.

Author Contributions: Conceptualization, methodology, software, formal analysis, investigation, data curation, writing—original draft preparation, D.S.; formal analysis, writing—review and editing, visualization, M.S.N.S.; formal analysis, investigation, S.S.; formal analysis, investigation, L.E.P.N.; formal analysis, investigation, C.F.B.; formal analysis, investigation, R.G.N.; software, formal analysis, writing—review and editing, visualization, B.B.d.M.; funding, visualization, R.C.d.S.; visualization, supervision, G.L.Z.; writing—review and editing, visualization, supervision, M.V.T.; writing—review and editing, visualization, supervision, project administration, M.A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Coordination for the Improvement of Higher Education Personnel (CAPES - 001), National Council of Technological and Scientific Development (CNPq - 306241/2020-0; 428180/2018-3), the Research Support Foundation of the State of Rio Grande do Sul (FAPERGS - 21/2551-0002253-1), and SoluBio[®] (03/2021).

Data Availability Statement: Not applicable.

Acknowledgments: This work was supported by the National Council of Technological and Scientific Development (CNPq) and the partner company SoluBio[®].

Conflicts of Interest: The authors declare no conflict of interest.

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