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Bioherbicide based on *Diaporthe* sp. secondary metabolites in the control of three tough weeds

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Brazilian growers are facing difficulties to manage the weeds *Lolium multiflorum*, *Conyza* sp. and *Echinochloa* sp. using commercially available agrochemicals in most crops. This study aims to evaluate the use of a bioherbicide based on fermented broth containing secondary metabolites of *Diaporthe* sp. to control *L. multiflorum*, *Conyza* sp. and *Echinochloa* sp. The bioherbicide activity in pre-emergence and post-emergence of these weed species and phytotoxicity on soybean, wheat and rice plants were evaluated. In the pre-emergence test using all weed species, the bioherbicide showed 100% control efficiency in comparison with the control group. Phytotoxic symptoms were observed on soybean leaves and horseweed was efficiently controlled using the bioherbicide.

Key words: Bioherbicide, weeds, bioproducts.

INTRODUCTION

Several factors can restrict the production yield in agricultural species and among them, weeds are a tough competitor, which usually can be controlled using different methods, such as mechanical, physical, biological and chemical (Silva and Silva, 2012). Combining different methods that reduce weed development and its effects on agricultural species is seen as a promising strategy (Nunes et al., 2010). However, weed management in recent decades has been accomplished mostly through chemical control (Oliveira et al., 2011), leading to the selection of resistant biotypes (Christoffoleti et al., 1997).

The high number of weed species resistant to herbicides calls for new modes of action and herbicide classes. However, an herbicide with a new mode of action has not been launched on the market in the last 20 years (Dayan and Duke, 2014). In this context, biological molecules are considered important sources to be explored for the development of new products for weed management (Duke et al., 2002). According to Duke et al. (2000) there are many toxins of natural origin still

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unexplored and which have herbicide action with different modes of action from those currently known. Fungi are one option and often are found in nature colonizing and causing severe damage in some species of weeds (Barreto and Evans, 1998). This has led to an increased number of studies using these microorganisms and their metabolites as weed control agents, as well as the discovery of new sites of action and bioactive molecules (Dayan and Duke, 2014). Several studies have shown herbicidal activity of metabolites produced by microorganisms on weeds. Varejão and Demuner (2013) identified compounds produced by *Alternaria euphorbiicola* showing herbicidal activity on *Euphorbia heterophylla*. Khattak et al. (2014) found metabolites of *Aspergillus* spp. and *Penicillium* spp. negatively interfering in the growth of *Lemna minor* and in the seed germination of *Silybum marianum*.

In a previous study, our research group collected thirty-nine phytopathogenic fungi from infected tissues of weeds and isolated them in the laboratory looking for the production of metabolites with herbicidal action. Among them, a strain belonging to the *Diaporthe* sp. genus was screened due to herbicidal effects of its secondary metabolites on target plants (Souza et al., 2016a). In a second study, the production of the bioherbicide in a bench-scale bioreactor with working volume of 3 L was assessed (Souza et al., 2015b). The bioherbicide efficiency rate reached 100% in pre-emergence, since there was no seed germination after its application. *Diaporthe* has been reported often as producing secondary metabolites for biological control of weeds (Ash et al., 2010; Andolfi et al., 2015). Phomentrioloxin B caused small necrotic spots on a number of plant species, whereas gulypyrone A caused leaf necrosis in *Helianthus annuus* plantlets (Andolfi et al., 2015). Cimmino et al. (2013) tested several compounds produced in liquid culture by *Phomopsis* sp. (teleomorph: *Diaporthe gulyae*) for the control of the annual weed *Carthamus lanatus*.

The main objective this study was to evaluate the application of fermented broth containing secondary metabolites of *Diaporthe* sp. to control *Lolium multiflorum*, *Conyza* sp. and *Echinochloa* sp. The herbicidal activity was evaluated in post-emergence and pre-emergence of these weed species and also on *Glycine max*, *Triticum aestivum* and *Oryza sativa.*

EXPERIMENTAL SECTION

Weeds and cultures used in the bioassays

Two bioassays were carried out to evaluate the herbicidal effect of secondary metabolites of *Diaporthe* sp. obtained by submerged fermentation in the pre-emergence of soybean (*Glycine max*), wheat (*Triticum aestivum*), rice (*O. sativa*), rye grass (*L. multiflorum*) and sorghum (*Sorghum halepense*) and in the post-emergence of soybean (*Glycine max*), wheat (*T. aestivum*), rice (*Oryza sativa*), rye grass (*L. multiflorum*), horseweed (*Conyza* sp.) and rice grass (*Echinochloa* sp.).

Location of bioassays

Pre-emergence assays were carried out in an incubator with controlled humidity (85%) and temperature (28 °C), while the post-emergence evaluation was carried out in a greenhouse, located at the coordinates 29° 43'39.79''S and 53°33'40.06''W, Santa Maria – Brazil.

Production of metabolites by submerged fermentation

The strain of *Diaporthe* sp. was isolated from plants of the weed *Solanum americanum* Mill in the Pampa biome and was used in this study for bioherbicide production by submerged fermentation. The strain of *Diaporthe* sp. was kept in a potato dextrose agar (PDA) at 4°C and subcultured every 15 days. Cell production for pre-inoculum was incubated in a Petri dish containing PDA for 8 days at 28ºC (Souza et al., 2016a). Afterwards, a 6 mm disc of fungal mycelium was transferred to a 250 mL Erlenmeyer flask, which contained 100 mL of fermentation medium at 28°C at 120 rpm for 7 days (Inova 44R, NewBrunswick) for inoculum. The fermentations were carried out in a batch bioreactor (BIOTEC-C, Tecnal, Brazil), containing 3.0 L of the optimized culture medium, using a 10% (v/v) inoculum at an initial pH of 6.0, incubated at 28°C for 7 days at 40 rpm. The culture medium was composed of corn steep liquor 33% (v/v), sucrose 18 $g.L^{-1}$, $(NH_4)_2SO_4$ 2 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.5 $g.L^{-1}$, $FeSO_4.7H_20$ 1 $g.L^{-1}$ and $MnSO_4.H_2O$ 1 $g.L^{-1}$. After fermentation, the cell mass was separated from the fermentation broth by centrifugation at 4000 rpm for 10 minutes and the supernatant used in the bioassays.

Herbicidal activity in pre-emergence

The activity of the bioherbicide obtained from *Diaporthe* sp*.* was evaluated in the pre-emergence of dicotyledon seeds (*G. max*) and monocotyledon seeds (*T. aestivum*, *O. sativa*, *L. multiflorum* and *S. halepense*). For this purpose, 10 mL of fermented broth without the cells were sprayed on a germitest paper containing 100 seeds of each culture and maintained at 28°C (POL-EKO, model KK) 350. A control test was carried out by replacing the fermented broth with distilled water. Seed germination was evaluated during 7 days, according to the Brazilian rules for seed analysis (Brasil, 2009). Afterwards, the germinated and non-germinated seeds were counted. All seeds that presented primary root protrusion higher than 2 mm were considered to be germinated.

Herbicidal activity in post-emergence

For the evaluation of herbicidal activity in post-emergence, seven different concentrations of fermented broth were sprayed on the upper part of *G. max, T. aestivum, O. sativa, L. multiflorum, Conyza* sp. and *Echinochloa* sp. plants. The concentrations were 0D (water), 1/4D, 1/2D, 1D, 2D, 4D and 8D, where D corresponds to undiluted fermented broth. A mineral oil 0.5% (v/v) was added to all treatments.

The experimental design was completely randomized with five replicates. The units were 500 ml polyethylene pots, filled with commercial substrate Macplant®. Four seeds of each species evaluated were manually sown in each pot. After plant emergence only one plant was maintained per pot. For the fermented broth sprays, a backpack sprayer was used, which was pressurized by CO_2, provided with a bar pattern with four tips, model Teejet XR

110.02, with a pressure of 40 Ibf and spacing between tips of 0.5 m. The walking speed during spray was 1 m s^{-1} and a volume of 200 L ha^{-1} was used. At spray time, the plants had between 2-3 leaves and the temperature and relative air humidity were 31.2°C and 62%, respectively.

In each treatment, shoot dry matter, weed control and injury according to Frans et al. (1986) were evaluated. The injury for the species *G. max*, *T. aestivum* and *O. sativa* was determined at 7 and 15 days after the fermented broth spray (DAA). The control of *L. multiflorum, Conyza* sp. and *Echinochloa* sp. was evaluated at 15 DAA. After the determination of control and injury at 15 DAA, the aerial part of plants was sectioned, packed in paper bags and dried at 70°C during 72 h to obtain shoot dry matter.

Statistical analysis

All data were submitted to analysis of variance and Tukey's test was used to verify significant differences between sprays ($p<0.05$) using the software Assistat® 7.7 beta.

RESULTS

Herbicidal activity in pre-emergence

Table 1 presents the herbicidal activity in pre-emergence of fermented broth. Seeds of *G. max*, *T. aestivum*, *O. sativa*, *L. multiflorum* and *S. halepense* did not present root protrusion. The germination inhibition reached 100% for all species, whereas in the control test, germination varied by species, but reached values higher than 60% (Figures 1 and 2).

Table 1. Pre-emergence activity of secondary metabolites of *Diaporthe* sp.

Species	Germinated seeds (%)	
	Fermented broth	Control
Lolium multiflorum	0	68
Triticum aestivum	0	78
Oryza sativa	0	95
Glycine max	0	87
Sorghum halepense	0	91

Herbicidal activity in post-emergence

The effect of fermented broth on shoot dry matter mainly for species of *Conyza* sp. and *Echinochloa* sp was exhibited in Figure 3. The best results for *Conyza* sp. were obtained when 4D and 8D were applied, however, plant death did not occur.

The effect of the bioherbicide on injury of crop species shown in Table 2. Only *G. max* presented injury symptoms, which was verified at 7 and 14 days after application (DAA) for concentrations higher than 2D and most severely for 8D. Plants showed symptoms, such as yellowing and blight on youngest leaves, whereas the oldest leaves showed partial necrosis at the tips, as presented in Figure 4.

DISCUSSION

Herbicidal activity in pre-emergence

These results confirm that metabolites of *Diaporthe* sp. have herbicidal activity in pre-emergence of some weed species, providing an important alternative for the management of weeds resistant to chemical herbicides. The pre-emergent control has the advantage of reducing competition from weeds with the crop especially in the initial phase of its establishment as a result of lower germination of weeds, besides enabling a reduction in the seed bank.

The metabolites of *Diaporthe* sp. showed a broad spectrum of action in pre-emergence, since activity was verified in both dicotyledonous (*G. max*) and monocotyledonous seeds (*T. aestivum*, *O. sativa*, *L. multiflorum* and *S. halepense*). Souza et al. (2015b) obtained an efficiency of 100% in the pre-emergence of *Cumumis sativus* (dicotyledonous) and *Sorghum* sp. (monocotyledonous) seeds when fermented broth of *Diaporthe* sp. was used. The control of different plant families, as well as different species, is considered beneficial from an agricultural viewpoint, since simultaneous growth of different species of weeds is common in crops and an herbicide should have the broadest action possible.

The inhibition of germination by the action of secondary metabolites of *Diaporthe* sp. may be related to molecules from the terpenoid groups which are produced by a wide range of fungi. Castro et al. (2001) reported that terpenoids have hormonal functions, inhibiting the growth and acting in active-transport through the membrane. However, the confirmation of this hypothesis should be based on more in-depth studies.

Herbicidal activity in post-emergence

In practice, the increased concentrations increased the amount of molecules with herbicide activity, which are very diluted in the fermentation medium (Varejão and Demuner, 2013). As a consequence, a suppressive effect was verified instead of control, as shown in Figure 5 for *Conyza* sp. For this weed, 4D and 8D resulted in about 50% reduction in weight of shoot dry matter, when compared with the control treatment. Even though significantly less, it can be seen that the fermented broth caused a reduction in the weight of shoot dry matter for *Echinochloa* sp. at all doses used. The 8D concentration of fermented broth also resulted in a significant reduction of weight of shoot dry matter for the *G. max* species. This confirms that phytotoxins are highly diluted in the fermentation broth, so that an effect only occurs when it is used at higher concentrations.

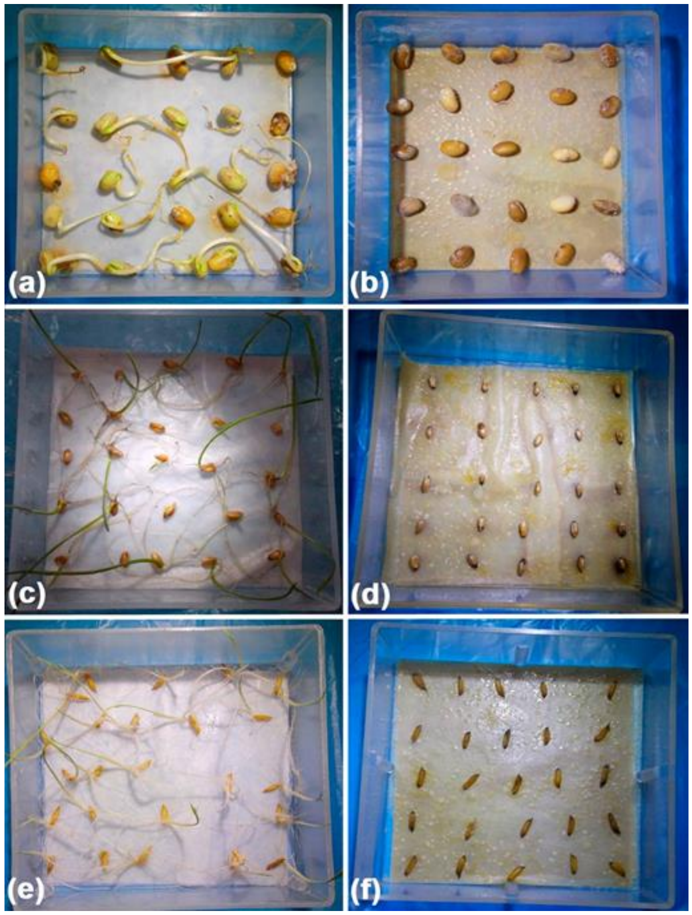

Figure 1. Germination of *Glycine max*, *Triticum aestivum*, *Oryza sativa* in the control test (a, c and e) and using bioherbicide fermented broth (b, d and f), respectively.

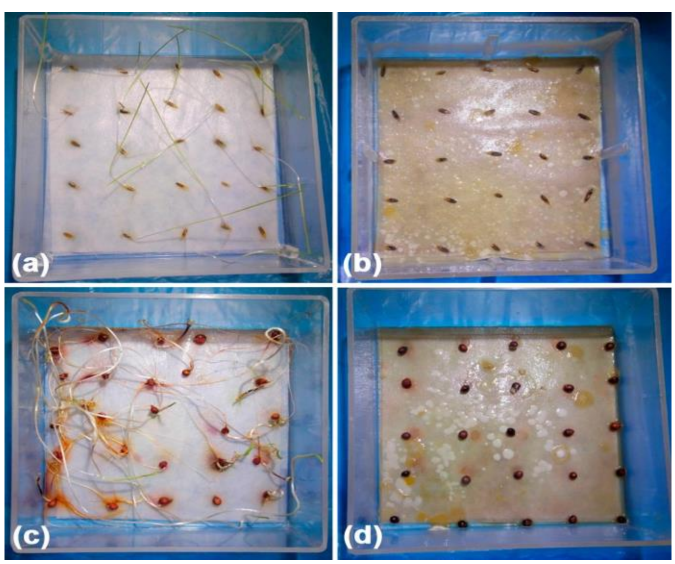

Figure 2. Germination of *Lolium multiflorum* and *Sorghum halepense* in the control test (a and c) and using bioherbicide fermented broth (b and d), respectively.

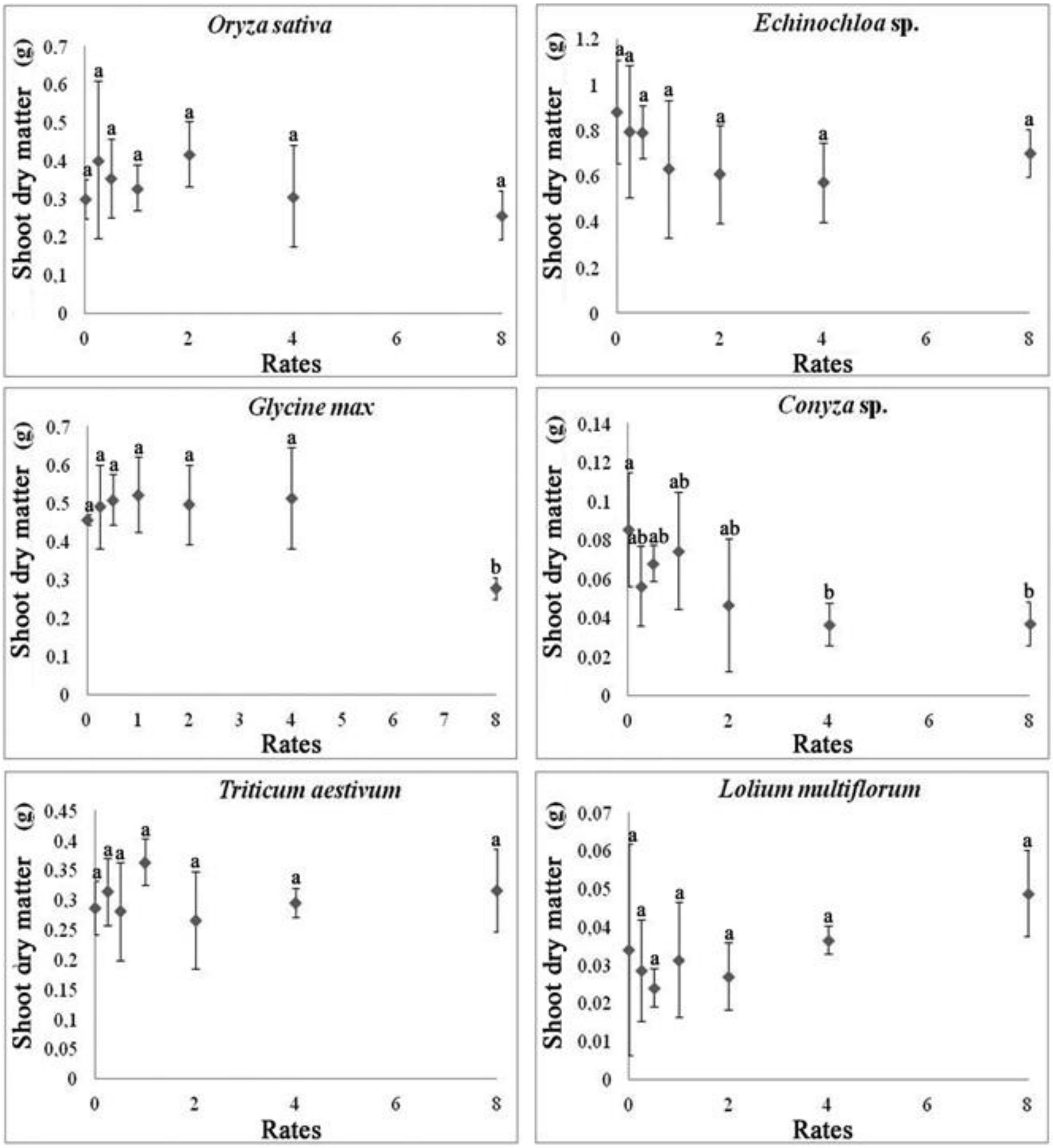

Figure 3. Influence of bioherbicide on shoot dry matter.

In addition, the plants presented significant shoot dry matter reduction as a result of injury caused by the application of fermentation broth. The observed symptoms suggest that phytotoxins present systemic action, as both *G. max* and *Conyza* sp. plants presented a retardation of plant growth. One hypothesis related to the retarded development of plants and consequent reduction of shoot dry matter may be related to interference of the phytotoxin in the biosynthesis of amino acids that are essential in plant growth, such as valine, leucine and isoleucine.

Conclusions

In this study, the application of fermented broth of *Diaporthe* sp. as a bio-herbicide in pre- and post-emergence of different crop species (*O. sativa*, *G. max* and *T. aestivum*) and weeds (*Echinochloa* sp., *Conyza* sp. and *L. multiflorum*) was investigated. In pre-emergence, the fermented broth showed an efficiency of 100%, since no seeds of any species germinated. In post-emergence, the most promising results were obtained for *Conyza* sp. and *Echinochloa* sp. species,

Table 2. Phytotoxic effect of bioherbicide on crop species.

Treatment	Phytotoxicity (%)	
	7 DAA	15 DAA
Glycine max		
0	0	0
¼ D	0	0
½ D	0	0
1 D	0	0
2 D	10	10
4 D	40	20
8 D	80	70
Triticum aestivum		
0	0	0
¼ D	0	0
½ D	0	0
1D	0	0
2 D	0	0
4 D	0	0
8 D	0	0
Oryza sativa		
0	0	0
¼ D	0	0
½ D	0	0
1D	0	0
2 D	0	0
4 D	0	0
8 D	0	0

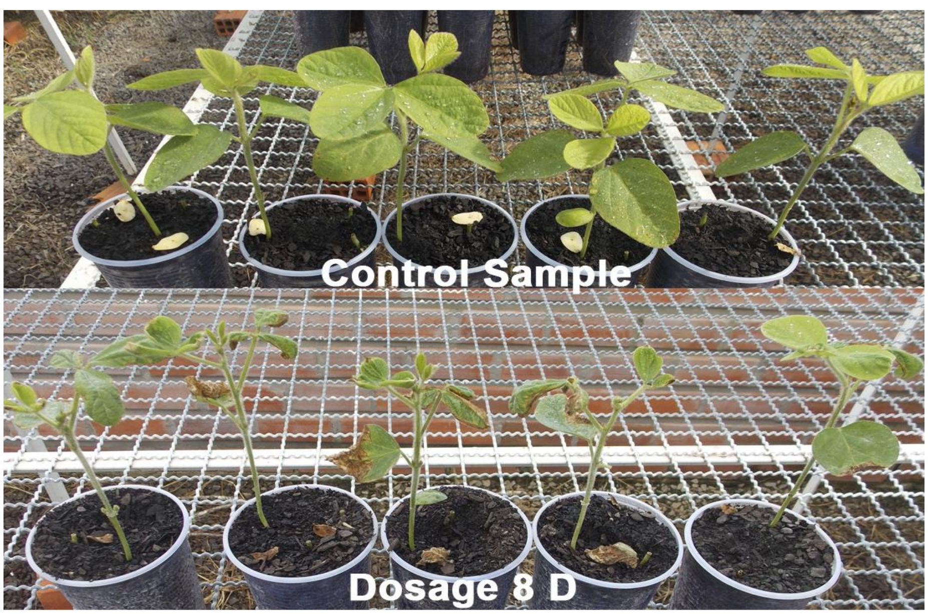

Figure 4. At the top of the figure, plants of *Glycine max* exempted from metabolites *Diaporthe* sp., at the bottom plants under the highest concentration of metabolites.

Figure 5. Influence of bioherbicide in the control of *Conyza* sp.

because plant growth was suppressed. *G. max* showed injury to metabolites when concentrations were higher than 2D at 7 and 15 DAA. The biomolecules produced by *Diaporthe* sp. are promising for as a natural herbicide.

Conflicts of Interests

The authors have not declared any conflict of interests.

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