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RHIZOBACTERIA ABLE TO PRODUCE PHYTOTOXIC METABOLITES

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ABSTRACT

To contribute for the development of environmental friendly methods for weed control, a selection of rhizobacteria able to produce phytotoxic substances was carried out. Initially, 35 strains previously isolated from plants in the south of Minas Gerais State (Brazil) were grown in tryptic soy broth. After removal of bacterial cells, the resulting liquids were freeze-dried and extracted with methanol/ethyl acetate (1:1). The extracts were concentrated under vacuum and dissolved in water to be submitted to a lettuce (*Lactuca sativa* L.) seed assay. Metabolites produced by five strains reduced the number of normal seedlings to values statistically bellow the one observed for the negative control, being the most expressive results obtained with *Bacillus cereus* Frankland and Frankland, isolated from *Ricinus communis* L., which was able to cause rotted rootlets to 82.4% of seedlings. The bacterium metabolites also avoided germination of 52% *Brachiaria decumbens* Stapf seeds and the remaining 48% resulted in abnormal seedlings. Metabolites from *B. cereus* were submitted to a purification process guided by the lettuce seed assay. As a consequence, one substance causing rotted rootlets to all lettuce seedlings during the seed assay at 0.057 g/L was isolated and will be identified in future studies.

Key words: *Bacillus cereus*, bioherbicide, *Lactuca sativa*, phytotoxin, rhizobacteria, *Brachiaria decumbens*

INTRODUCTION

Weed control is so important for agriculture that 40-60% of all worldwide commercialized agrochemicals correspond to herbicides. Although these substances are essential to obtain the necessary food and fiber, they also present some negative aspects as the increase of cost production and the contamination of human beings and the environment with toxic residues (13,23,24). Consequently, methodologies less expensive and toxic to control weed are greatly welcome. Apparently such a problem can be solved by the use of rhizobacteria, which may affect the development of plants with a magnitude as large as the one observed for phytopathogenic bacteria (20). As an example it is possible to mention the use of *Pseudomonas putida* (Trevisan) Migula, *Stenotrophomonas maltophilia* Palleroni and Bradbury and *Enterobacter taylorae* Hormaeche and Edwards to control *Bromus tectorum* L. in wheat fields (22), or the application of *Pseudomonas* spp. in corn fields to control *Striga hermonthica* (Del.) Benth. (1).

As it has been observed that rhizobacteria can act against plants by the production of phytotoxic substances like cyanide (2), indole-3-acetic acid (21) and haterumalide A (12), a simple method to identify rhizobacteria potentially useful for weed control could consist on submitting their metabolites to an assay with lettuce seed (*Lactuca sativa* L.), which has been described as an excellent phytotoxin detector (31). Thus, this work was aimed to use this assay to select phytotoxin producing rhizobacteria to be employed in future studies directed to the development of new methods for weed control.

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MATERIALS AND METHODS

Production of rhizobacterial metabolites

Rhizobacteria used in this study are on deposit at the Department of Plant Pathology - Federal University of Lavras, State of Minas Gerais, Brazil. They were previously isolated from roots of signalgrass (*Brachiaria* sp.), coffee (*Coffea arabica* L.), okra (*Hibiscus esculentus* L.), indian cress (*Tropaeolum majus* L.), tomato (*Lycopersicon esculentum* Mill.), marigold (*Tagetes erecta* L.), castorbean (*Ricinus communis* L.), pigeonpea (*Cajanus cajan* L.), common bean (*Phaseolus vulgaris* L.), corn (*Zea mays* L.) and lettuce (*Lactuca sativa* L.) by Coimbra (8), who identified the microorganisms by fatty acid methyl esters analysis, which were carried as described elsewhere (7, 19). They were grown on tryptic soy agar (TSA - Merck KgaA) during two days, at 28ºC, and transferred to tryptic soy broth culture medium (TSB - Isofar Indústria e Comércio de Produtos Químicos). After ten days at 28ºC, under constant stirring (100 cpm), bacterial cells were removed by centrifugation (10,000 g) and 70 mL of each supernatant liquid were freeze-dried. To each resulting residue were added 14 mL of a methanol/ethyl acetate (MeOH/AcOEt; 1:1) solution and the mixture obtained was filtered through cotton. The filtrates were concentrated to dryness in a rotary evaporator and dissolved in distilled water (70 mL) to be submitted to the lettuce seed assay.

Lettuce seed assay

Fifty (twenty five when pure substance was assayed) lettuce seeds (*Lactuca sativa* L. cv. Salad Bowl) were disposed in a transparent plastic box (GERBOX – 11.4 x 11.4 cm) containing two sheets of germination paper (10.5 x 10.5 cm) embedding an amount of sample to be evaluated equivalent to 2.5 times their weight. After incubation at 20ºC under a 12 h photoperiod, during seven days, the following parameters were used to evaluate the experiment: non-germinated seeds and seedlings with rotted rootlets (6). As negative controls distilled water and/or TSB extract obtained as described for rhizobacteria supernatant liquids were employed. Aqueous solutions of glyphosate at 6.85 mg/L, 17.14 mg/L and 34.28 mg/L, which were prepared by dissolving in water Agrisato 480 CS, produced by Alkagro do Brasil Ltda, were used as positive controls. The experiment was carried out with four replicates, in a randomized design. Results were directly submitted to analysis of variance, and average values were compared by Scott-Knott (27) calculations ($P \leq 0.05$). Statistical analyses were done using SISVAR software (11).

Signalgrass seed assay

Seeds of signalgrass (*Brachiaria decumbens* Stapf), produced by Sempra Sementes Ltda (Brazil), were soaked in concentrated sulfuric acid during 15 min and washed with water (6). Then, they were employed in an assay adapted from the lettuce seed assay described above for the crude rhizobacteria metabolites. Temperature was changed to 35ºC during the exposure to light and to 25ºC during the remaining period. The following parameters were used to evaluate the experiment: up to the fifth day non-germinated seeds and abnormal seedlings (with rotted rootlets, inexistent root, shoots shorter than 1.4 cm or without leaves) (6).

Purification of *Bacillus cereus* Frankland and Frankland metabolites

B. cereus was grown in TSA and some colonies were transferred to eight flasks containing 250 mL of TSB each. After 11 days at 25ºC, under constant stirring (100 cpm), cells were removed by centrifugation (10,000 g) during 15 min and supernatant liquids were combined and freeze-dried. Part (0.69 g) of the resulting mass (39.33) was successively washed with AcOEt (2 x 7 mL) and MeOH/AcOEt (1:1; 2 x 14 mL). All fractions were concentrated under reduced pressure and dissolved in 35 mL of distilled water to be submitted to the lettuce assay.

Another part (20 g) of the freeze dried metabolites was washed with AcOEt/MeOH (1:1; 6 x 100 mL) and the liquid phase was concentrated under reduced pressure to dryness. It resulted in a residue (7.52 g) from which an aliquot (1.0 g) was taken and passed through a silica gel column (2.3 x 15.0 cm) with AcOEt, MeOH, H_2O and 0.1 M HCl (200 mL of each). From the four new fractions samples amounting to 30% of their volume were removed, concentrated under reduced pressure, dissolved in 35 mL of H_2O and submitted to the lettuce assay. The remaining (70%) of the MeOH fraction was concentrated under reduced pressure to afford a residue (0.514 g) that was eluted through a silica gel column (2.3 x 8.0 cm) with AcOEt/MeOH (50 mL, 9:1; 50 mL, 4:1; 50 mL, 7:3; 50 mL, 3:2; 50 mL, 1:1), MeOH (100 mL), H_2O (100 mL) and 0.1 M HCl (100 mL). Samples corresponding to 40% of the resulting fractions were concentrated under reduced pressure and dissolved in 35 mL H_2O to be submitted to the lettuce assay. The remaining (60%) of the AcOEt/MeOH (9:1) fraction was concentrated under reduced pressure to afford a residue (0.004 g) that was analyzed by thin layer chromatography (TLC) employing plastic plates coated with silica gel 60. AcOEt/MeOH (9:1) was used to elute the sample and spots were visualized with ultraviolet light, iodine vapor and ceric sulphate solution (3). Then, the residue (0.004 g) was passed through a silica gel column (0.8 cm x 15.0 cm) with AcOEt/hexane (100 mL, 1:1), AcOEt (40 mL) and MeOH (50 mL). Fractions of 5 mL were collected and analyzed by TLC as described above. Five new fractions were obtained by combination of fractions 1-2, 3-16, 17-19, AcOEt and MeOH. Samples amounting to 50% of each of them were concentrated under low pressure and dissolved in 35 mL of H_2O to be submitted to the lettuce seed assay. The remaining of the AcOEt fraction was concentrated

under low pressure to afford a white solid weighting 0.002 g. It was analyzed by TLC as described before, employing AcOEt/hexane (1:1) as eluent.

A simplified scheme of the procedures employed in the purification steps is presented on Fig. 1.

RESULTS

Among the 35 rhizobacteria strains studied, only the metabolites produced by *Bacillus cereus*, *B. pumillus* (strain 55-30), *Enterobacter asburiae*, *E. cloacae* (strain 54-10) and *Microbacterium liquefaciens* afforded numbers of normal seedlings statistically bellow those observed for TSB and water, which were the negative controls (Table 1). The phytotoxic effects were observed mainly on the rootlets, whose size were smaller than those obtained for healthy seedlings and usually presented rotted regions.

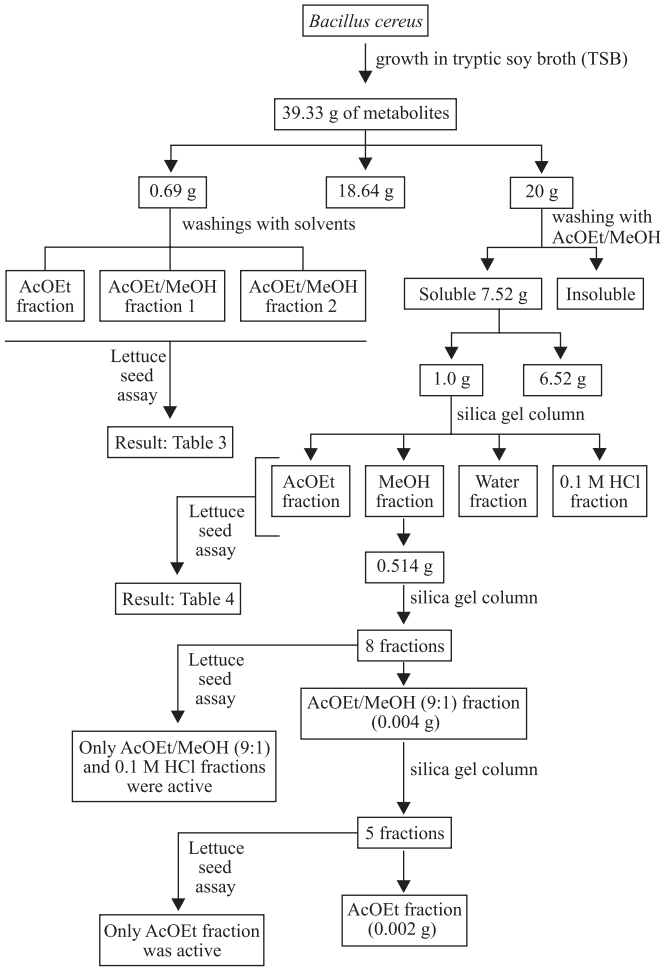

Figure 1. Simplified scheme of the *Bacillus cereus* metabolites purification.

As the most expressive results were observed for the metabolites produced by *B. cereus*, isolated from *Ricinus communis*, they were also used against *Brachiaria decumbens* seeds. The number of healthy seedlings was considerably reduced (Table 2).

When crude *B. cereus* metabolites were submitted to small scale solvent washings, it was observed that AcOEt could not dissolve the active substance produced by that bacterium (Table 3). Thus, it was necessary to employ mixtures of such solvent and MeOH to extract the phytotoxic metabolite.

During the first chromatographic process, only those fractions eluted with MeOH and 0.1 M HCl presented toxic effects to lettuce seeds. Actually, no seed exposed to them could germinate (Table 4).

When the MeOH fraction (Table 4) was submitted to a new chromatographic process, eight new fractions were obtained (AcOEt/MeOH 9:1; 4:1; 7:3; 3:2; 1:1, MeOH, H_2O and 0.1 M HCl), among which the most active was the one eluted with AcOEt/MeOH (9:1) that caused rotted rootlets to 98% of seedlings. It was also observed that the fraction eluted with 0.1 M HCl presented a very weak phytotoxic effect, while all the other fractions were inactive against lettuce seeds.

During the last purification step, which was carried out by a chromatographic fractionation of the AcOEt/MeOH (9:1) fraction, only the material eluted with AcOEt was active. It caused rotted rootlets to 93% of seedlings. When analyzed by TLC, only one homogenous spot could be observed for the solid resulting from such fraction.

DISCUSSION

Although metabolites produced by five rhizobacteria strains have presented phytotoxic activity during the lettuce seed assay, only *Bacillus cereus* afforded really expressive values (Table 1). The other four microorganisms metabolites presented results very near to the negative controls (TSB and water). Such behavior is in accordance with the work by Karadeniz *et al.* (16), who found out that *B. cereus* could produce indole-3-acetic acid, giberelic acid, zeatin and abscisic acid when grown in brain heart broth. All these substances are plant growth hormones, whose effect on plant usually varies with their concentrations (26).

The literature reveals that *Bacilus pumillus* can be used in bioherbicide formulations (14). However, only strain 55-30 presented some activity against lettuce seeds (Table 1). Perhaps that is a consequence of indole-3-acetic acid production, since Kang *et al.* (15) have observed that such bacterium is able to synthesize this substance in tryptophan-amended medium.

Although *Bacillus megaterium* is able to inhibit *Ipomoea hederacea* Jacq. growth through the production of phytotoxins (16,17), its metabolites presented no effect on lettuce (Table 1). A possible explanation for these results could rely on the AcOEt

Table 1. Effect of rhizobacteria crude metabolites on lettuce seeds.

Rhizobacteria	Plant source	Normal seedlings[1,2]	Non-germinated seeds[1, 2]
Agrisato 480 CS (concentration 01)[3]		45.5b	1.5a
Agrisato 480 CS (concentration 02)[4]		0a	2.0a
Agrisato 480 CS (concentration 03)[5]		0a	2.0a
Bacillus cereus Frankland and Frankland (strain 57-02)[6]	*Ricinus communis* L.	0a	8.7c
Bacillus megaterium de Bary (strain 54-06)	*Coffea arabica* L.	48.3e	0.7a
B. megaterium (strain 55-16)	*Hibiscus esculentus* L.	46.0d	1.7a
B. megaterium (strain 55-21)	*Coffea arabica* L.	48.0e	1.5a
Bacillus pumillus Meyer and Gottheil (strain 54-08)	*Trapaeolum majus* L.	49.3e	0.2a
B. pumillus (strain 54-23)	*Hibiscus esculentus* L.	46.3d	2.5a
B. pumillus (strain 55-13)	*Coffea arabica* L.	49.3e	0.7a
B. pumillus (strain 55-30)	*Lycopersicon esculentum* Mill.	42.6c	5.2b
B. pumillus (strain 62-20)	*Lactuca sativa* L.	46.0d	2.5a
B. pumillus (strain 83-03)	*Zea mays* L.	47.5e	0.5a
B. pumillus (strain 83-10)	*Zea mays* L.	47.0d	1.5a
B. pumillus (strain 83-11)	*Zea mays* L.	48.1e	0.7a
B. pumillus (strain 83-16)	*Zea mays* L.	48.1e	1.7a
B. pumillus (strain 83-17)	*Zea mays* L.	47.8e	1.2a
B. pumillus (strain 83-18)	*Lycopersicon esculentum* Mill.	46.5d	2.5a
B. pumillus (strain 84-27)	*Brachiaria* sp.	46.1d	1.2a
Bacillus sphaericus Meyer and Neide (Strain 62-18)	*Lactuca sativa* L.	49.0e	0.5a
Bacillus thurigiensis Berliner (strain 84-12)	*Brachiaria* sp.	46.6d	1.2a
B. thurigiensis (strain 55-15)	*Tagetes* sp.	47.8e	1.0a
B. thurigiensis (strain 57-25)	*Phaseolus vulgaris* L.	47.1d	2.2a
Enterobacter asburiae Brenner *et al.* (strain 62-04)	*Lactuca sativa* L.	40.3b	6.0b
Enterobacter cloacae Hormaeche and Edwards (strain 54-01)	*Coffea arabica* L.	47.0d	3.0a
E. cloacae (strain 54-10)	*Trapaeolum majus* L.	43.8c	3.5a
E. cloacae (strain 55-05)	*Coffea arabica* L.	49.3e	0.2a
E. cloacae (strain 55-07)	*Coffea arabica* L.	48.1e	1.2a
E. cloacae (strain 57-29)	*Lycopersicon esculentum* Mill.	48.8e	0.7a
E. cloacae (strain 58-20)	*Lactuca sativa* L.	46.6d	1.7a
E. cloacae (strain 58-24)	*Lactuca sativa* L.	48.1e	1.2a
Enterobacter hormaechei Hormaeche and Edwards (strain 62-01)	*Lactuca sativa* L.	46.6d	1.7a
Klebsiella planticola Bagley, Seidler and Brenner (strain 56-29)	*Lycopersicon esculentum* Mill.	46.0d	1.0a
Klebsiella pneumoniae (Schroeter) Trevisan (strain 57-15)	*Cajanus cajan* L.	45.8d	2.2a
Kluyvera cryocrescens Farmer *et al.*	*Trapaeolum majus* L.	47.1d	2.2a
Microbacterium liquefaciens Takeuchi and Hatano	*Coffea arabica* L.	39.0b	6.2b
Paenibacillus macerans Ash *et al.* (strain 62-12)	*Lactuca sativa* L.	49.0e	0.7a
Stenotrophomonas maltophilia Palleroni and Bradbury	*Lycopersicon esculentum* Mill.	45.6d	3.2a
TSB[7]		45.0d	2.0a
Water		45.3d	4.0b

[1]Average values after seven days at 20ºC; [2]Values with the same letter in each column do not differ from each other statistically according to Scott and Knott (27) calculations at 5% probability; [3]6.85 mg of glyphosate/L; [4]17.14 mg of glyphosate/L; [5]34.28 mg of glyphosate/L; [6]Necrosis on roots; [7]after freeze drying and extraction.

Table 2. Effect of *Bacillus cereus* crude metabolites on *Brachiaria decumbens* seeds.

Treatments	Abnormal seedlings[1,2]	Non-germinated seeds[1]
B. cereus metabolites	24.0b	26.0a
TSB[3]	09.2a	23.7a
Water	10.0a	24.2a

[1]Means of four replicates with the same letter in a column do not differ significantly ($P \leq 0.05$) according to the Scott-Knott (27) calculations; [2]Normal plantlets average height was 2.8 cm; [3]After extraction with AcOEt/MeOH (1:1).

Table 3. Effect on lettuce seeds of fractions obtained during the small scale washings of the *Bacillus cereus* crude metabolites.

Fractions[1]	seedlings with rotted rootlets[2]	non-germinated seeds[2]
AcOEt	07a	00a
AcOEt/MeOH 1	70c	30b
AcOEt/MeOH 2	31b	69c
Water (control)	02a	01a

[1]AcOEt (ethyl acetate); MeOH (methanol); [2]Means of four replicates with the same letter in a column do not differ significantly ($P \leq 0.05$) according to the Scott-Knott (27) calculations.

Table 4. Effect on lettuce seeds of fractions obtained during the first chromatographic fractionation of the *Bacillus cereus* metabolites.

Fractions[1]	seedlings with rotted rootlets[2]	non-germinated seeds[2]
AcOEt	06a	02a
MeOH	01a	99b
Water (fraction)	09a	05a
0.1M HCl	00a	100b
Water (control)	07a	02a

[1]AcOEt (ethyl acetate); MeOH (methanol); [2]Means of four replicates with the same letter in a column do not differ significantly ($P \leq 0.05$) according to the Scott-Knott (27) calculations.

lack of capability for dissolving some substances (28). Perhaps, *B. megaterium* had produced phytotoxic metabolites in TSB medium that could not be dissolved in AcOEt/MeOH and, consequently, did not get in contact with lettuce seeds. To circumvent such possible problem, in a preliminary work AcOEt was completely substituted with MeOH, which possesses an increased capacity to dissolve different substances (28). However, the results were not satisfactory because the TSB MeOH extract, which was one of the negative controls, considerably affected lettuce.

Although *Enterobacter hormaechei* presented no activity, the only strain of *Enterobacter asburiae* reported here and *Enterobacter cloacae* (strain 54-10) affected lettuce in a very low extension (Table 1). The last bacterium can produce the plant growth regulators phenylacetic acid, indole-3-acetic acid and tyrosol in liquid media (29), while the ability of the other two microorganisms to produce phytotoxic substances is unknown. It is also worth of mention that *Enterobacter intermedius* Hormaeche & Edwards can produce 3-methylthiopropanoic acid, which can affect weed seedling development (18), and that *Enterobacter taylorae* Hormaeche & Edwards can suppress germination and seedling growth of *Bromus tectorum* L. (22).

Stenotrophomonas maltophilia can either be used as a plant growth promoter (4) and as *Bromus tectorum* L. germination and seedling growth suppressor in wheat fields (22). According to Park *et al.* (25) and Suckstorff and Berg (32), it is also able to produce 3-indole-acetic acid. Anyway, *S. maltophilia* presented no effect against lettuce (Table 1). Similarly, other plant growth promoting rhizobacteria, *Kluyvera cryocrescens* (34) and *Kluyvera pneumoniae* (10, 16), were inactive against lettuce seeds.

Concerning *Bacillus sphaericus*, *B. thuringiensis*, *Paenibacillus macerans*, *Klebsiella planticola*, *K. cryocrescens* and *M. liquefaciens*, the absence of phytotoxic activity for their metabolites (Table 1) seemed reasonable, since no report describing any deleterious effect of such bacteria on plant was found.

As *B. cereus* metabolites presented the most promising results during the lettuce seed assay, it was submitted to a test with seeds from *Brachiaria decumbens*, a weed that causes considerable losses in Brazilian coffee production (30). Although the number of germinated seeds did not differ statistically from the one observed for the negative control, no healthy seedlings could be obtained (Table 2). This result seems in accordance with Zaidi *et al.* (33) and Karadeniz *et al.* (16), who described the ability of *Bacillus* spp. to produce plant hormones, which can present deleterious effects on plants (26).

In order to contribute for the development of a new method for weed control employing *B. cereus*, a preliminary study was carried out to identify the metabolite active against *B. decumbens*. During the small scale solvent washings, the intermediate polarity of the active substance was evident, since AcOEt (5) could not dissolve it (Table 3, Fig. 1).

Surprisingly, when metabolites soluble in AcOEt/MeOH (1:1) were eluted through silica gel, two active fractions were obtained (Table 4, Fig. 1), indicating the production of at least two phytotoxins by *B. cereus*. When the MeOH fraction (Table 4) was submitted to another chromatographic process, eight new

fractions were obtained, among which the AcOEt/MeOH (9:1) and 0.1 M HCl fractions presented deleterious effects on lettuce seeds. As it seems very probable that the active substance eluted with MeOH during the first chromatographic process would elute with MeOH or water during the following step (9), apparently the microorganism produced one substance that decomposed to another one, much more polar, which eluted with 0.1 M HCl.

During the last fractionation on silica gel (Fig. 1), the only active fraction, eluted with AcOEt, seemed to correspond to a pure substance, since it presented a very homogeneous spot when analyzed by TLC (3). In the near future, such substance will be identified and produced in a large scale to be submitted to testes with *B. decumbens* to evaluate its potential to control this weed.

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RESUMO

Rizobactérias produtoras de substâncias fitotóxicas

Com vistas a contribuir para o desenvolvimento de métodos não agressivos ao meio ambiente, para o controle de plantas invasoras, buscou-se selecionar rizobactérias produtoras de substâncias fitotóxicas. Inicialmente, 35 culturas previamente isoladas de plantas da região sul do Estado de Minas Gerais (Brasil) foram cultivadas em caldo soja tripticaseína. Após remoção das células bacterianas, os líquidos resultantes foram liofilizados e extraídos com metanol/acetato de etila (1:1). Os extratos foram concentrados sob vácuo e submetidos a testes com sementes de alface (*Lactuca sativa* L.). Os metabólitos produzidos por cinco isolados bacterianos reduziram o número de plântulas normais para valores estatisticamente inferiores aos observados para o controle negativo, sendo os mais expressivos resultados obtidos com *Bacillus cereus* Frankland and Frankland, isolado de *Ricinus communis* L., que causou necroses nas radículas de 82,4% das plântulas. Os metabólitos dessa bactéria também impediram a germinação de 52% das sementes de *Brachiaria decumbens* Stapf e fizeram com que as 48% restantes dessem origem a plântulas anormais. Os metabólitos de *B. cereus* foram submetidos a processos de purificação direcionados por testes com sementes de alface. Em decorrência, isolou-se uma substância, que será identificada em estudos futuros. Na concentração de 0,057 g/L, causou necrose nas radículas de todas as plântulas de alface provenientes do teste com sementes.

Palavras-chave: *Bacillus cereus*, bio-herbicida, *Lactuca sativa*, fitotoxina, rizobactéria, *Brachiaria decumbens*

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