Research Article

Glyphosate-induced impact on the functional traits of the *Bacillus* sp. FC1 isolate[1]

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ABSTRACT

The intensive use of glyphosate has shown to be harmful to non-target organisms. Considering the soil as a final herbicide acceptor, the microbial community present on it is one of the critical factors to be monitored. This study aimed to isolate *Bacillus* sp., as well as to evaluate the effect of glyphosate on functional traits related to the growth and biocontrol activity of the phytopathogen. *Bacillus* sp. FC1 was isolated from the soil and grown in six media, in the presence and absence of glyphosate. The analysis of bacterial growth consisted of comparing the growth curves. The effect of glyphosate on the biocontrol activity was evaluated by antibiosis against the phytopathogen *Fusarium* sp. Glyphosate had a negative effect on the growth of the *Bacillus* sp. FC1 isolate. Exposure to the herbicide, based on the antibiosis method, showed no effect on the biocontrol activity of the phytopathogen. However, the sensitivity of the isolate to the herbicide may have affected its ability to initially compete for nutrients with the phytopathogen in the medium.

KEYWORDS: Biomonitoring, biocontrol, crop-beneficial soil bacteria, herbicide tolerance.

RESUMO

Impacto induzido pelo glifosato nas características funcionais do isolado *Bacillus* sp. FC1

O uso intensivo de glifosato tem se mostrado prejudicial para organismos não-alvos. Considerando-se o solo como um dos receptores finais do herbicida, a comunidade microbiana presente é um dos fatores críticos a serem monitorados. Objetivou-se isolar *Bacillus* sp. e avaliar o efeito do glifosato nas características funcionais do crescimento e na atividade de biocontrole do fitopatógeno. *Bacillus* sp. FC1 foi isolado do solo e cultivado em seis tratamentos, na presença e ausência de glifosato. A análise de crescimento bacteriano consistiu na comparação das curvas de crescimento. O efeito do glifosato sobre a atividade de biocontrole foi avaliado por antibiose contra o fitopatógeno *Fusarium* sp. O glifosato apresentou efeito negativo no crescimento do *Bacillus* sp. FC1. A exposição ao herbicida, baseada no método de cultura pareada, não afetou a característica de biocontrole do fitopatógeno. Contudo, a sensibilidade do isolado ao glifosato pode ter afetado a capacidade inicial de competição por nutrientes com o fitopatógeno em placa.

PALAVRAS-CHAVE: Biomonitoramento, biocontrole, bactérias benéficas do solo, tolerância a herbicidas.

INTRODUCTION

Glyphosate [(N-phosphonomethyl) glycine] is one of the most widely used herbicides for weed control, which targets the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) required for the synthesis of aromatic amino acids in the shikimate pathway (Gill et al. 2017). EPSPS is a precursor for the primary and secondary metabolisms of plants and many microorganisms; thus, its inhibition may affect the synthesis of a large number of secondary metabolites, including antifungal metabolites (Tzin & Galili 2010). However, even though this herbicide may have a low contamination potential, its extensive use has shown to be harmful to non-target organisms (Brito et al. 2017).

Studies have shown the effects of glyphosate on the soil microbial community (Newman et al. 2016, Van Bruggen et al. 2018), whose knowledge is important to understand how the shifts on ecological and functional traits of such communities, when under herbicidal effects, could lead to the loss of valuable ecosystem services (Newman et al. 2016). Therefore, Van Bruggen & Semenov (2000) suggest a biomonitoring approach to the soil health by measuring bacteria responses to various stresses.

In the present study, *Bacillus* sp. rhizobacteria were used as a biological parameter. *Bacillus* sp. is a diverse and common soil bacterial species, ubiquitous

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and broadly adapted to grow in diverse environments. It can be isolated in greater numbers from a variety of rhizospheres (Earl et al. 2008). In addition, *Bacillus* sp. is one the most related crop-beneficial soil bacterial genera and plays an essential role in several ecological processes, such as promoting growth and inducing systemic resistance in plants, and as a biocontrol agent by producing a broad range of antimicrobial compounds (Shafi et al. 2017, Lopes et al. 2018).

Although studies on the interaction between herbicides and soil microorganisms seek to understand biodegradation processes, the impact of herbicides on their functional traits has been poorly documented. Therefore, the present study aimed to isolate *Bacillus* from the soil and evaluate the effect of glyphosate on functional traits related to growth and phytopathogen biocontrol activity.

MATERIAL AND METHODS

The experiment was carried out at the Instituto Federal Goiano, in Rio Verde (Goiás state, Brazil), from August to December 2016.

Soil samples were obtained from the rhizosphere of carvoeiro (*Tachigali vulgaris*), a native Brazilian Savannah tree, collected at the Universidade de Rio Verde, in Rio Verde, Goiás state, Brazil (17º46'59.2"S and 50º57'59.5"W). About 10 g of soil of each sample were homogenized in 90 mL of peptone water (H_2Op) and treated in a water bath at 80 ºC, for 12 min. Serial dilution in H_2Op was performed at a concentration of 10^{-1}, 10^{-2} and 10^{-3} bacteria and inoculated (100 µL) in nutrient agar. The selected isolates were put aside for further morphological and biochemical studies (Buchanan & Gibbons 1975). Bacterial DNA extraction was performed using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. In addition, a partial sequence of the 16S rRNA gene was amplified with primers R1387 (5'- CGG TGT GTA CAA GGC CCG GGA ACG - 3') (Heuer et al. 1997) and P027F (5' - GAG AGT TTG ATC CTG GCT -3') (Lane et al. 1985). Sequencing analysis was conducted using an ABI 3130 Genetic Analyzer (Applied Biosystems), aligned by ClustalW in Mega 7 (Kumar et al. 2016), and then the aligned sequence was used for the construction of a phylogenetic tree based on maximum parsimony.

The Universidade de Rio Verde provided potassium salt of glyphosate - under the trade name Roundup Transorb R® (containing 480 g L^{-1} of the active ingredient of glyphosate). Minimal medium (MM) was defined as the basal medium for the experiment. The medium, in g L^{-1} of distilled water and pH (7.0), was composed of KH_2PO_4 (1.5), Na_2HPO_4 (0.6), NaCl (0.5), NH_4SO_4 (2), $MgSO_4$ $7H_2O$ (0.2), $CaCl_2$ (0.01) and $FeSO_4$ $7H_2O$ (0.001) (Dworkin & Foster 1958). Nutrient broth (NB) was also used, being composed, in g L^{-1}, of peptone (10), beef extract (10) and NaCl (5). The media with the herbicide were supplemented with 7.2 mg mL^{-1} of glyphosate. The dextrose concentration in the minimal medium dextrose (MMD) and minimal medium dextrose herbicide (MMDH) treatments was 1 % (w/v).

The isolated strains were grown aerobically in NB at 27 ºC, for 36 h. The bacterial culture was standardized to 1 % of the original ($\lambda = 0.8$ at 600 nm). The selected strain was grown in 250 mL Erlenmeyer flasks containing 50 mL of six media in the presence and absence of glyphosate and incubated in the dark on a rotary shaker (100 rpm) at 27 ºC, for 168 h. The bacterial growth was monitored by measuring the optical density (OD) in a spectrophotometer, at a wavelength of 600 nm, while the cell viability was measured by colony-forming unit (CFU). After 15 h of exposure to the treatments, the samples (100 µL) were diluted in a physiological solution containing 0.85 % NaCl and loaded in triplicate onto nutrient agar plates incubated at 30 ºC, for 24 h. The data expressed in CFU mL^{-1} were converted to Log CFU mL^{-1}.

For the antibiosis assay after the exposure to glyphosate, the selected isolated strain was grown in 4-L Erlenmeyer flasks containing 1.3 L of nutrient broth and incubated in the dark on a rotary shaker (150 rpm) at 27 ºC. The cell extraction was performed during the exponential phase. The culture was centrifuged at 5,000 rpm, for 5 min. The pellets were homogenized in a physiological solution of 0.85 % NaCl and separated into aliquots for the following treatments: MMD, MMDH, NB and NBH (NB supplemented with herbicide). After 15 h of exposure to the treatments, the cultures were centrifuged at 5,000 rpm, for 5 min, and subsequently washed. The cells obtained from each treatment were suspended in a physiological solution and transferred to sterile Petri dishes. A sterilized potato dextrose agar (PDA) medium was cooled to 40~50 ºC, cell suspension was

added, rapidly shaken, and poured into a Petri dish (Pour plate). After the medium solidification, 5-mm diameter mycelial discs of *Fusarium* sp. were placed on the center of the PDA plate and then incubated at 25 ºC in the dark. The antibiosis dishes (*Bacillus* sp. FC1 after exposure to treatments and to *Fusarium* sp.) and the control dish (*Fusarium* sp. growing without *Bacillus* sp. FC1) were incubated for two weeks and then the mycelial growth of the phytopathogens was evaluated.

The percentage of growth inhibition was calculated using the formula (R1-R2)/R1 x 100, where R1 is the radial distance of the *Fusarium* sp. mycelium in the absence of the antagonist from the center to the edge of the plate (measured in mm) and R2 is the growth distance of *Fusarium* sp. from the center of the plate to the bank toward *Bacillus* sp. FC1. Each treatment was repeated three times. All experiments had a completely randomized design and were conducted in three replicates for each treatment. The significance of the observed differences was verified using one-way analysis of variance (Anova) followed by the Tukey test. All statistical analyses were performed using the R Studio software (R Core Team 2017).

RESULTS AND DISCUSSION

Several morphologically different colonies were isolated and purified. Based on the morphological, structural and biochemical characterizations, the FC1 isolate exhibited similarity to the *Bacillus* genus. The isolate also produced amylase, catalase, acetyl methyl carbinol (acetoin) from glucose fermentation, citrate utilization, tolerance to high temperature, and salinity (Table 1).

By 16S rRNA partial gene sequencing (905 bp), the isolate displayed similarity (96 %) with *Bacillus* sp. A phylogenetic tree was generated using *Bacillus* strains closely related to the FC1 isolate by comparison of the 16S rRNA sequence to the National Center for Biotechnology Information (NCBI) database. The FC1 isolate is related, clustered, with *Bacillus* sp. (KR259192.1) (Figure 1). Based on the results of the biochemical tests, 16S rRNA partial gene sequence and the phylogenetic tree, it was concluded that the FC1 belonged to a *Bacillus* species.

Table 1. Morphological, structural and biochemical tests conducted for the *Bacillus* sp. FC1 isolate identification.

Tests	Results
Morphology	Rod shape
Gram staining	+
Sporulation	+
Starch hydrolysis	+
Voges Proskauer	+
Methyl red	-
Citrate utilization	+
Growth in 6.5 % NaCl	+
Growth at 50 ºC	+
Catalase	+

(+) positive; (-) negative.

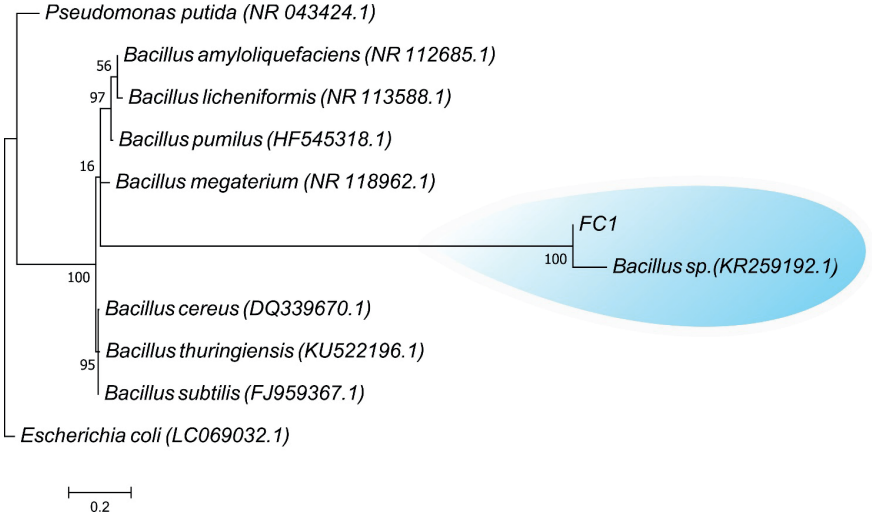

Figure 1. Maximum-parsimony phylogenetic tree constructed from the 16S rRNA sequence of the *Bacillus* sp. FC1 isolate and related sequences obtained from the National Center for Biotechnology Information (NCBI) database. The GenBank accession number for each strain is shown in parentheses. *Escherichia coli* (LC069032.1) was used as an outgroup.

Previously, a screening curve was determined to analyze the bacterial behavior at first exposure to glyphosate (Figure 2). The *Bacillus* sp. FC1 growth was dramatically affected in the presence of glyphosate, with the highest growth level being 0.060 nm, for 168 h of incubation. Differential growth in response to medium constitutions revealed significant differences ($p < 0.05$) between the treatments.

With the screening curve, it was possible to determine the glyphosate effect on the growth of the *Bacillus* sp. FC1. However, the screening curve only showed the OD averages in the decline phase, which occurred between 24 h and 48 h - visible on the MMD graph bars (Figure 1). Furthermore, the minimal medium represents a simple medium without any alternative carbon source, except for MMD and MMDH, which does not provide sufficient energy to allow for cellular propagation (Martins et al. 2011). Additionally, auxiliary carbons increase the biodegradation potential of the bacterial culture by increasing its metabolic activity (Kumar & Philip 2006). In view of that, a complex medium (NB, nutrient broth) was added so as to investigate whether such a low growth was due to the medium composition.

Glyphosate also affected the growth of the *Bacillus* sp. FC1, when cultured in the media containing a complex carbon source (NBH). As shown in Figure 3, the highest growth level was observed in

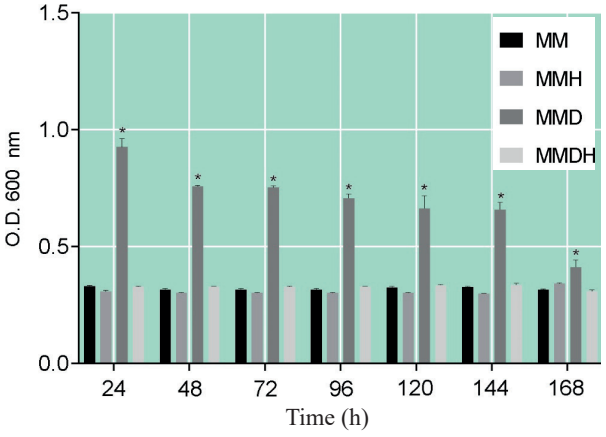

Figure 2. Screening curve of the *Bacillus* sp. FC1 in the absence (control) and presence of glyphosate, for 168 h of incubation. The results represent the means obtained with data from the replicates (n = 3). * Means are significantly different ($p < 0.05$) by the analysis of one-way variance (Anova) and the Tukey test. MM: minimal medium; MMH: minimal medium herbicide; MMD: minimal medium dextrose; MMDH: minimal medium dextrose herbicide.

the control media (NB and MMD). Glucose is often used as a carbon source for bacteria, in order to enhance their growth and metabolism, present in dextrose, MMD and MMDH. However, the *Bacillus* sp. FC1 was unable to use it as a sole carbon source in the presence of glyphosate. This result could be attributed to the inhibition of the enzyme EPSPS, essential in the shikimate pathway, responsible for the biosynthesis of aromatic amino acids in plants and microorganisms (Schulz et al. 2006). Aristilde et al. (2017) showed that aromatic amino acid supplementation in the growth medium leads to the recovery of metabolic homeostasis in soil *Pseudomonas* species, when cultured with herbicide. Their finding supports the inhibition of aromatic amino acids by the EPSPS interruption in the shikimate pathway.

The cell viability data showed a strong concordance with growth kinetics (Figure 3), as shown in Figure 4. After exposed to glyphosate for 15 h, though relatively low in the treatments with herbicide, an increase in the number of viable cells was observed, if compared to the concentration of the initial inoculum (Figure 4). This result suggests that glyphosate has a bacteriostatic effect on the *Bacillus* sp. FC1 growth (i.e., temporarily inhibits the bacterium growth). The low metabolic rate on the MMDH and NBH treatments can be a mechanism to protect the cell against the toxic effects of the herbicide (Gray et al. 2019), resulting in an herbicide tolerance mechanism. Martins et al. (2011) also reported the effects of herbicides on soil bacteria and showed the activation of antioxidant enzymes as the herbicide tolerance mechanism.

The *Bacillus* sp. FC1 was 95 % effective in the growth inhibition of *Fusarium* sp., which did not differ between treatments in the presence and absence of glyphosate (data not shown). However, a clear zone surrounding the mycelia of the *Fusarium* sp. and FC1 was detected in both culture media treatments (Figure 5).

It was investigated whether the mycelial growth produced a larger halo on the *Bacillus* sp. FC1 lawn in the glyphosate treatments (NBH and MMDH) than that in the control treatment (NB and MMD). However, in terms of occurrence, both treatments varied in clearer and well-defined halos during the incubation (Table 2).

After two weeks of incubation, it was observed a late mycelial growth of *Fusarium* sp., which overlapped the halos in the MMDH and NBH

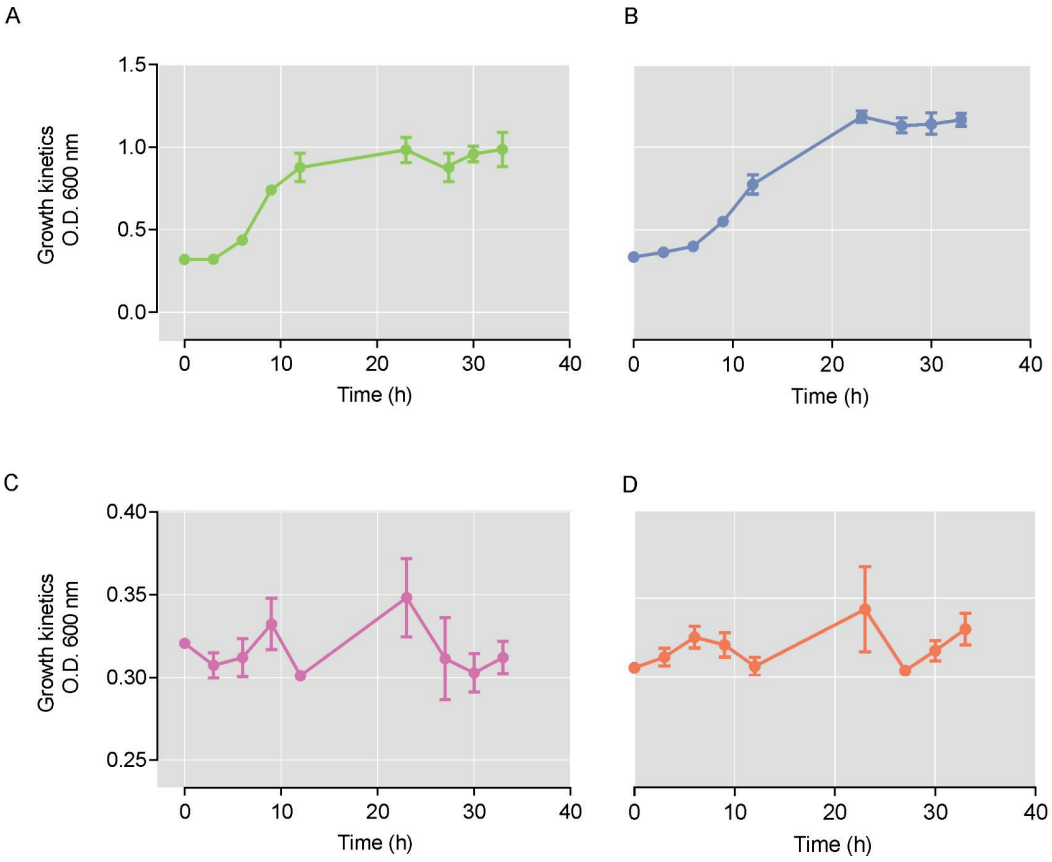

Figure 3. Growth kinetics of the *Bacillus* sp. FC1 in the absence and presence of glyphosate, for 33 h of incubation. A) nutrient broth; B) minimal medium dextrose; C) nutrient broth herbicide; D) minimal medium dextrose herbicide. Results represent the means obtained with data from the replicates (n = 3).

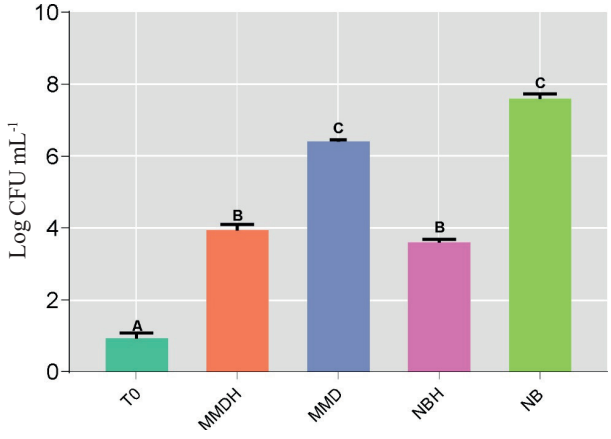

Figure 4. Cell viability after exposure to glyphosate for 15 h, measured by colony-forming unit (Log CFU mL^{-1}). The values represent the means from three experiments ± SEM. Means with different letters are significantly different ($p < 0.05$) by one-way analysis of variance (Anova) and the Tukey test. T0: initial inoculum; NB: nutrient broth; NBH: nutrient broth herbicide; MMD: minimal medium dextrose; MMDH: minimal medium dextrose herbicide.

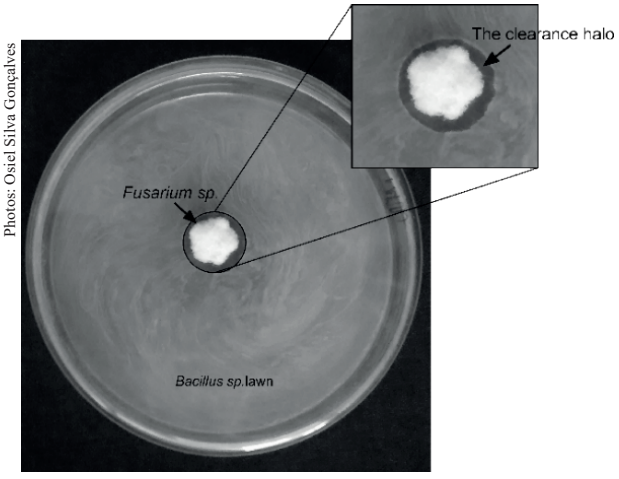

Figure 5. Co-culture interspecific interaction showing the clear zone surrounding the mycelia of *Fusarium* sp. and the *Bacillus* sp. FC1 lawn. *Bacillus* sp. FC1 cell suspension, previously exposed to glyphosate for 15 h, was spread on a PDA plate with an incubated mycelial disc of *Fusarium* sp. The plates were incubated at 25 ºC in the dark, for two weeks.

plates; whereas *Fusarium* sp. cultured in MMD and NB formed a clear zone surrounding the mycelium (Table 2 and Figure 6).

No evidence was found supporting any toxic secondary metabolites of *Fusarium* sp. over *Bacillus* sp. Therefore, it was assumed that the formation of halos on the plates was due to the disposition of the mycelial discs in the media, with *Fusarium* sp. previously competing for space and resources, thus excluding the bacterial cell growth (i.e., competitive exclusion) (Hardin 1960). Remarkably, an important aspect was revealed with the inversion of halos after two weeks of incubation. It is believed that the well-defined halos on the plates of treatments MMD and NB corresponded to the bacterium antagonistic action over the fungus (i.e., an inhibition halo) (Figures 6C and 6D). However, *Fusarium* sp. overlapped the halos on the plates in MMDH and NBH, what may indicate the sensitivity of the *Bacillus* sp. FC1 to the herbicide, supported by cell viability data (Figure 4), which initially affected its ability to compete with the phytopathogenic fungus.

Ahmad et al. (1995) also reported the influence of the bioherbicide phosphinothricin on interactions between phytopathogens and their antagonists, including *B. subtilis*, *Pseudomonas flourescens* and many *Trichoderma* species. According to their results, phosphinothricin has a strong impact on antagonistic bacteria, with *F. oxysporum* being able to expand over the entire plate area, when paired with either *B. subtilis* or *P. fluorescens*. The *Bacillus* species are also natural root-associated bacteria that can enhance plant growth by phosphate solubilization, ACC deaminase activity, production of siderophores and phytohormones (Souza et al. 2015). In this context, glyphosate could also have an adverse impact on functional traits, regarding plant growth.

The present study demonstrated that the *Bacillus* sp. FC1 decreases its population in response to glyphosate. These slow-growing bacteria tend to be less sensitive to glyphosate, resulting in an herbicide tolerance mechanism. Furthermore, there was also an experimental attempt to investigate the potential effect of the herbicide on the *Bacillus* sp. biocontrol, based on the antibiosis method against *Fusarium* sp. The hypothesis was that glyphosate could affect the biosynthesis of antifungal metabolites, considering the shikimate acid pathway disruption to be a precursor for a large number of secondary metabolites. Glyphosate did not show a marked inhibition of the *Bacillus* sp. FC1 antagonistic action over *Fusarium* sp. However, the sensitivity of the *Bacillus* sp. FC1 to the herbicide may affect its ability to compete with phytopathogenic fungi. These results provide insights into the interaction between herbicides and soil microorganisms, as well as perspectives for further studies to better understand how these compounds could affect bacterial functional traits.

Table 2. Halo occurrence in the treatments after glyphosate exposition.

Treatments	1st week incubation	2nd week incubation
MMD	+/-	+
MMDH	+	-
NB	+/-	+
NBH	+	-

MMD: minimal medium dextrose; MMDH: minimal medium dextrose herbicide; NB: nutrient broth; NBH: nutrient broth herbicide. (-) No halo; (+) strong halo; (+/-) moderate halo.

CONCLUSIONS

1. Glyphosate affects the growth of the *Bacillus* sp. FC1 isolate;

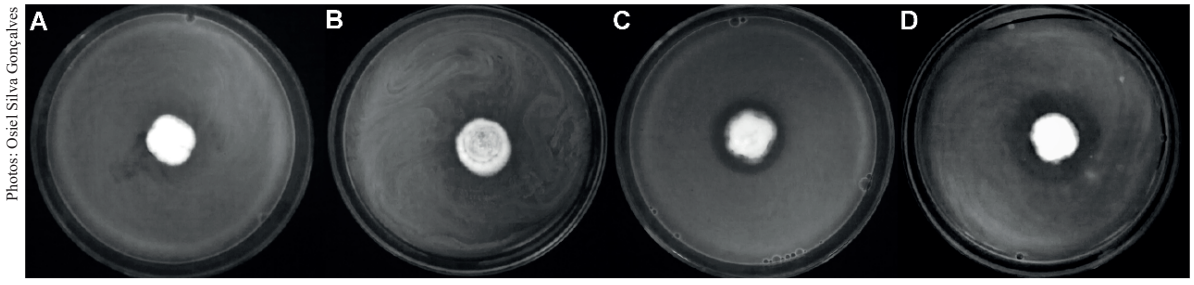

Figure 6. Antibiosis plates after two weeks of incubation evincing the inversion of halos with the *Bacillus* sp. FC1 in the following treatments: A) minimal medium dextrose herbicide; B) nutrient broth herbicide; C) minimal medium dextrose; D) nutrient broth. The results represent the data from the replicates (n = 3).

2. No effect of glyphosate on the *Bacillus* sp. FC1 biocontrol activity was detected.

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