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Bacillus subtilis – capacity for enzymatic degradation, resistance to trace elements, antagonisms and siderophore production

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Abstract

The use of microorganisms in agriculture as biofertilizers and biocontrol agents, in addition to their use in biotechnological practices, has been explored increasingly frequently over the years. Some bacteria, including *Bacillus subtilis*, have many capabilities related to promoting plant growth. The present study attempted to evaluate eight *B. subtilis* strains regarding their capacity for enzymatic degradation, resistance to trace elements, antagonism against phytopathogenic fungi and siderophore production. The tests were performed in plate dishes and test tubes with six repetitions for each bacterial isolate. The results showed that all isolates were able to perform enzymatic degradation to phosphatase, amylase and cellulase. Regarding resistance to trace elements, for Cd, 0.5 mmol L^{-1} was sufficient to prevent the development of strains 248, 263 and 320; for Cu, isolate 263 obtained greater resistance; for Zn, isolate 320 was inhibited at 2.0 mmol L^{-1}, for Cr(III), isolates 290 and 291 showed greater resistance to the metal, whereas for Cr(VI), isolates showed the same resistance pattern; and for Ni, isolates showed the same resistance behavior. *In vitro* antagonism occurred for all isolates; however, the antagonism occurred at different intensities, except for isolate 291. The production of siderophores was identified for only six isolates: 287, 320, 309, 274, 263 and 248. These results establish a foundation for further investigations to clarify the conditions and/or characteristics required by isolates for a more effective performance, observing metabolic routes and genetic mechanisms.

Keywords: Bioremediation, *Bacillus subtilis*, Biotechnology, Siderophores, Antagonism.

Introduction

The capacity to promote plant growth has been exhibited by many microbes. The beneficial effects of bacteria derived from the plant rhizosphere on roots and overall plant growth have been demonstrated. These types of bacteria have been designated plant-growth-promoting rhizobacteria (PGPR). The significant beneficial effect of these rhizobacteria on plant growth is achieved by both direct and indirect mechanisms. Direct methods include the production of compounds that stimulate plant growth and ameliorate stress (Goswami et al., 2016)

Plant rhizospheres are special environments with complex plant root-soil microbe interactions (Jha et al., 2013). These complex interactions are proposed to follow root exudations, which serve to attract beneficial soil bacteria to plant roots (Zhang et al., 2017).

Bacilli are among the most investigated rhizobacterial species (Souza et al., 2015), after Pseudomonas, mostly for their biocontrol activities (Idris et al., 2007). Reports indicate that bacilli are also the most abundant in plant rhizospheres (Sivasakthi et al., 2014), constituting up to 95% of the gram-positive rhizobacterial populations in plant rhizospheres (Prashar et al., 2013). According to Kumar et al. (2012a), these bacteria are efficient PGPR and capable of enhancing plant growth through the production of substances such as antibiotics and antifungal metabolites (Chowdhury et al., 2013), siderophores (Compant et al., 2005) and lytic enzymes (Nelson, 2004). Members of the Bacillus genus are particularly popular candidates for PGP because they sporulate and are easier to subject to commercial formulation (Mendis et al., 2018)

Bacillus species can form long-lived, stress-tolerant spores and secrete metabolites that stimulate plant growth and prevent pathogen infection (Radhakrishan et al., 2017).

Bacillus subtilis also plays a significant role in improving tolerance to biotic stresses. This induction of disease resistance involves the expression of specific genes and hormones such as 1-aminocyclopropane-1-carboxylate deaminase (ACC). Ethylene limits root and shoot growth and helps to maintain plant homeostasis. The degradation of the ethylene precursor (ACC) by bacterial ACC helps to relieve plant stress and maintain normal growth under stressful conditions (Glick et al., 2007).

Colonization of roots by *Bacillus subtilis* is beneficial to both the bacterium and the host plant. Approximately 30% of the fixed carbon produced by plants is secreted through root exudates. Colonization of the roots by bacteria provides a nutrient source, and in exchange, plants are the recipient of bacterial compounds and activities that stimulate plant growth and provide stress protection to their hosts. *Bacillus subtilis* forms a thin biofilm on the roots for long-term colonization of the rhizosphere. Root (Allard et al., 2016).

However, *B. subtilis* isolates demonstrated variable parameters related to promoting plant growth, and within each parameter, the isolates showed different levels.
Therefore, the present study aimed to characterize and identify the enzymatic degradation capacity, resistance to trace elements, antagonism against phytopathogenic fungi and production of siderophores demonstrated by eight *B. subtilis* strains.

Results

Enzyme degradation capacity
The results for the detection of different enzymes synthesized by *B. subtilis* isolates can be seen in S1. Under the established conditions, all isolates were able to produce phosphatase (Figure S1-a), amylase (Figure S1-b), and cellulase (Figure S1-c).
The test to identify inorganic phosphate solubilization (Figure S1-a) was positive for all isolates. *B. subtilis* was able to solubilize phosphate by presenting a halo around the colony.
For the production of amylase (Figure S1-b), the BHB medium enriched with starch was observed to be slightly opaque, and it was possible to visualize the halo without staining, as there was formation of a translucent area close to the culture; however, for the best definition and visualization of the halo, Lugol staining was performed. All isolates were able to produce amylase.
For the production of cellulase, all isolates were able to hydrolyze the CMC substrate, showing cellulolytic activity through the presence of halos around the colony (Figure S1-c).

Ability to resist trace elements
The existence of microorganisms in contaminated places or with the accumulation of trace elements is related to the resistance mechanism they present, which shows adaptation to such local conditions. When presenting resistance, these microorganisms multiply and become the majority, as they use polluting elements as a source of nutrients for their own survival. Thus, the 8 isolates identified as *Bacillus subtilis* were evaluated for resistance to Cu, Zn, Cd, Cr, Ni and $K_2Cr_7O_7$ (Figure 1).
Through Figure 1, it was possible to identify that isolates presented different types of resistance against the six trace elements under study. A low cadmium concentration (0.5 mmol L^{-1}) (Fig 1-d) was sufficient to prevent the development of 3 isolates - 37.5% of strains analyzed (248, 263, and 320). However, for zinc and chromium concentrations, isolates showed greater resistance, with IC_{50} values between 1.81 and 4.30 (Fig 1 - b/e). The lower the IC_{50} values were, the less resistant the microorganisms were to trace elements. At concentrations greater than 2.5 mmol L^{-1} for nickel, potassium dichromate, and copper ions, there was a significant decrease in the number of isolates that could be developed *in vitro*.
For the trace element $CuSO_4 \cdot 5H_2O$ (Fig 1-a), isolate 263 showed greater resistance at a concentration of 3.0 mmol L^{-1}; in contrast, isolates 297 and 309 were sensitive at concentrations above 2.0 mmol L^{-1}. For $ZnSO_4 \cdot 7H_2O$ (Fig 1-b), isolates 248, 274, 287, 290 and 309 were not affected by any concentration used; only isolate 320 showed the highest sensitivity at 2.0 mmol L^{-1} when compared to the others. When resistance to $K_2Cr_7O_7$ (Fig 1-c) was analyzed, 2.5 mmol L^{-1} was observed to be the concentration that most affected the development of isolates. Isolates resistant to $CdCl_2 \cdot H_2O$ (Fig 1-d) did not exceed concentrations above 1.5 mmol L^{-1}, with isolates 248 and 263 being affected at 0.5 mmol L^{-1}, followed by isolate 274, which showed sensitivity at 1.0 mmol L^{-1}.
For $CrCl_3 \cdot 6H_2O$ (Fig 1-e), isolates 290 and 291 were not affected by any of the concentrations used, since the other isolates (except 274) showed sensitivity at concentrations above 3.5 mmol L^{-1}. For the trace compound $NiCl_2 \cdot 6H_2O$ (Fig 1-f), all isolates were also observed to show the same tolerance/resistance and were affected at concentrations above 2.5 mmol L^{-1}.
Through mathematical calculations using the Weibull model, it was possible to obtain values for scale (beta) and shape (alpha) parameters, in addition to obtaining the statistical significance values (p-values) and the IC_{50} values, which allowed the identification of strains that were more sensitive to the metals used (Table 1). The values of parameters in bold indicate that they were statistically significant for the model used ($p < 0.05$). In the analysis of Zn and Cr, empty columns indicate that all isolates were able to grow under all tested concentrations (0.5 - 5.0 mmol L^{-1}). For trace element Cd, empty columns indicate that isolates were sensitive at all concentrations.

Correspondence analysis of resistance to trace elements
Figure 2 corresponds to the biplot formed by the correspondence analysis that is equivalent to the principal component analysis (PCA) for nonparametric statistics, identified by the first two principal components (CP1 and CP2), observing that the sum of the variability retained in these components is 93% of the original variability, of which CP1 and CP2 each have 70.1% and 22.9%, respectively. The projection on a two-dimensional plane, the biplot, seeks to specify the behavior of variables and contemplate sensitivities between isolates and trace elements. Isolates that are close to the direction of variables indicate lower sensitivity to the metal and, in the opposite direction, higher sensitivity.
The purpose of using this tool is to show the most relevant information from a data table to facilitate data presentation as new common variables called principal components. The data set was arranged in a matrix with nine lines corresponding to samples and thirty-nine columns corresponding to metals and their different concentrations.
In Figure 2, it is possible to perform a correlation with Figure 2 and to identify that isolates 320, 263 and 248 were extremely sensitive to Cd, being at the opposite end of the biplot. In correlation with Figure 1-d, basically all isolates were observed to be sensitive to this metal, since the highest IC_{50} values corresponded to 1.81. For Zn, isolates 263, 320, and 291 were also found at the opposite end of the biplot, demonstrating that they were affected by the different concentrations of the tested metal, with IC_{50} values of 1.81 for isolates 320 and 3.16 for isolates 263 and 320 (Figure 1-b). Isolates 290, 287, 309, 274, and 263 showed lower sensitivities to Ni (IC_{50} 2.65), Cr (IC_{50} 3.66 - 4.30), $K_2Cr_2O_7$ (IC_{50} 2.15 - 2.65) and Cu (IC_{50} 1.81 - 3.16).

In vitro antagonism against phytopathogenic fungi
The antagonistic effect of these B. subtilis isolates was evaluated against four phytopathogenic fungi, *Alternaria alternata, Colletotrichum gloeosporioides, Phyllosticta citricarpa, and Geotrichum citri-aurantii, in two culture media, BDA and King's B (Table 2).*

Table 1. Statistical data using the Weibull model for different concentrations of trace elements.

Trace elements	Parameters	Isolates							
		274	320	263	248	291	290	309	287
$CuSO_4 \cdot 5H_2O$	alfa	-28.88	-23.60	-34.16	-23.60	-28.88	-28.88	-35.35	-35.35
	beta	2.62	2.12	3.12	2.12	2.62	2.62	1.79	1.79
	IC50	2.65	2.15	3.16	2.15	2.65	2.65	1.81	1.81
	R2	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
$ZnSO_4 \cdot 7H_2O$	alfa		-35.35	-34.16		-34.16			
	beta		1.79	3.12		3.12			
	IC50		1.81	3.16		3.16			
	R2		0.9999	0.9999		0.9999			
$K_2Cr_7O_7$	alfa	-28.88	-23.60	-23.60	-23.60	-23.60	-23.60	-23.60	-23.60
	beta	2.62	2.12	2.12	2.12	2.12	2.12	2.12	2.12
	IC50	2.65	2.15	2.15	2.15	2.15	2.15	2.15	2.15
	R2	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
$CdCl_2 \cdot H_2O$	alfa	-7.60				-35.35	-35.35	-35.35	-35.35
	beta	0.60				1.79	1.79	1.79	1.79
	IC50	0.63				1.81	1.81	1.81	1.81
	R2	0.9999				0.9999	0.9999	0.9999	0.9999
$CrCl_3 \cdot 6H_2O$	alfa	-23.60	-39.44	-39.44	-39.44			-39.44	-39.44
	beta	4.24	3.62	3.62	3.62			3.62	3.62
	IC50	4.30	3.66	3.66	3.66			3.66	3.66
	R2	0.9999	0.9999	0.9999	0.9999			0.9999	0.9999
$NiCl_2 \cdot 6H_2O$	alfa	-28.88	-28.88	-28.88	-28.88	-28.88	-28.88	-28.88	-28.88
	beta	2.62	2.62	2.62	2.62	2.62	2.62	2.62	2.62
	IC50	2.65	2.65	2.65	2.65	2.65	2.65	2.65	2.65
	R2	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999

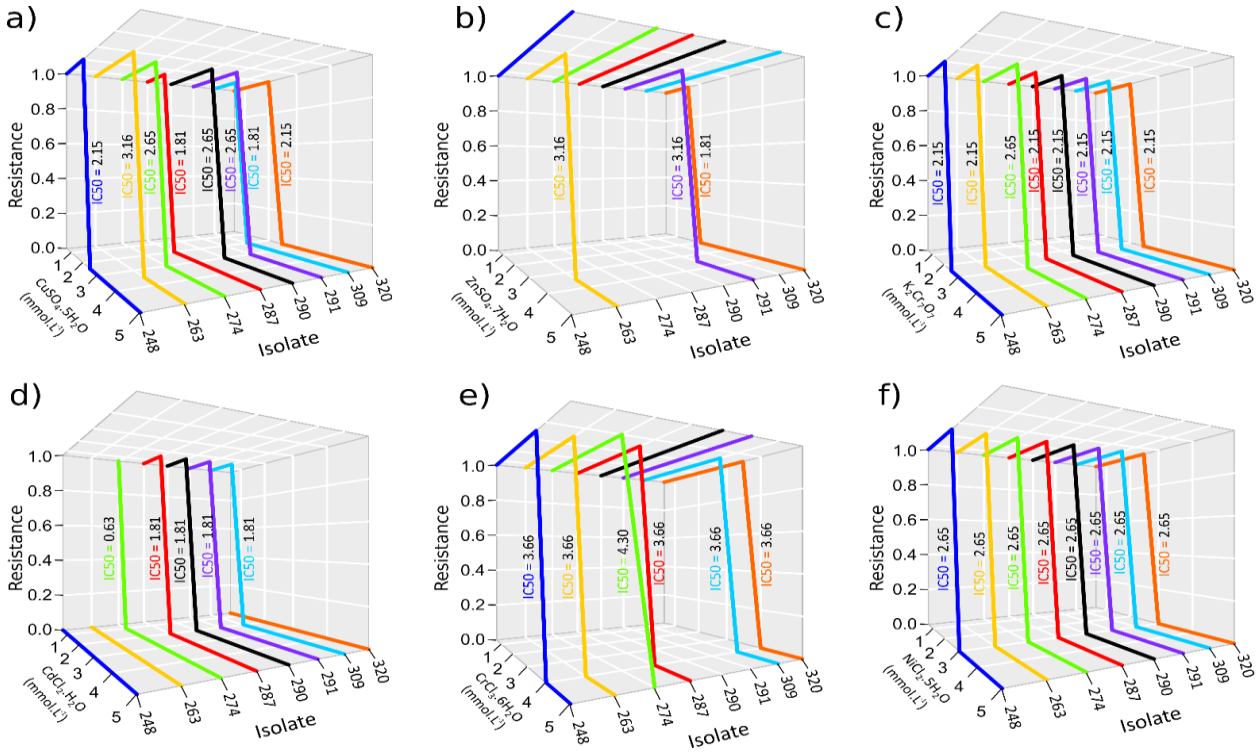

Figure 1. *B. subtilis* isolates resistant to concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0 mmol L-1 Cu, Zn, Cd, Cd, Cr, Ni and $K_2Cr_7O_7$. IC_{50} values indicate the minimum inhibitory concentration of 50%.

Table 2. Presence (+) or absence (-) of *in vitro* antagonism against fungi *Alternaria alternata, Colletotrichum gloeosporioides, Phyllosticta citricarpa*, and *Geotrichum citri-aurantii* in BDA and King's B culture media.

Fungus	*Alternaria alternata*		*Colletotrichum gloeosporioides*		*Phyllosticta citricarpa*		*Geotrichum citri-aurantii*	
	Culture medium							
Isolates	BDA	B de King	BDA	B de King	BDA	B de King	BDA	B de King
274	+	+	+	+	+	++	+	+
320	+	+	+	+	+	++	+	+
263	+	+	+	+	+	++	+	+
248	+	+	+	++	+	++	+	+
291	+	+	+	++	+	++	+	+
290	+	-	-	+	+	+	+	-
309	+	+	+	+	+	++	+	++
287	+	+	+	++	+	++	+	++

+ → weak antagonism, with the fungus avoiding growing on the bacterial colony; ++ → strong antagonism, presence of halo containing the fungus; - → no antagonism, with fungal growth on the bacterial colony.

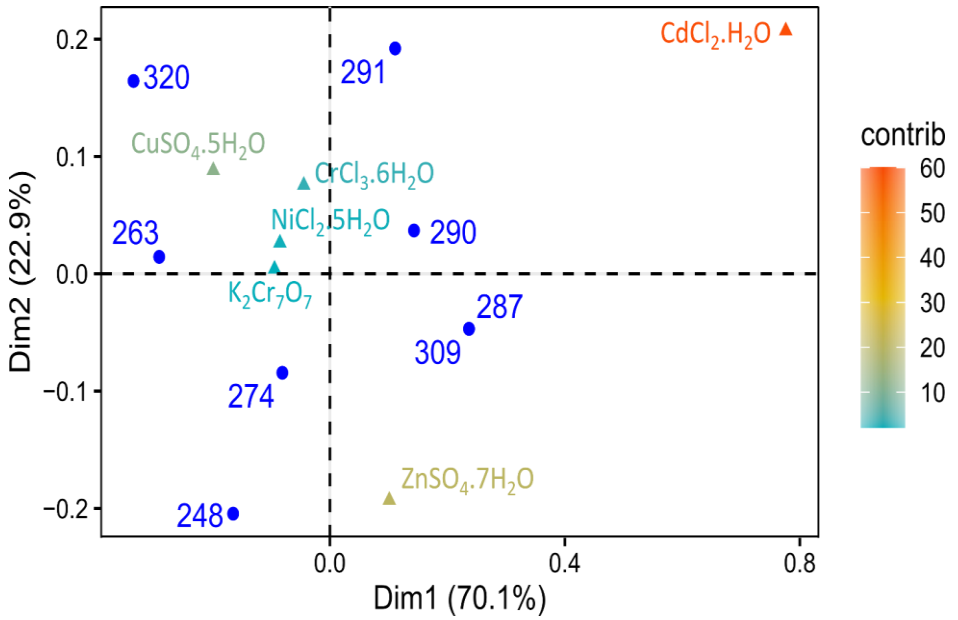

Figure 2. Spatial projection, with biplot, of principal components (PCs) regarding the sensitivity of the eight isolates to six trace elements - Cu, Zn, Cd, Cr, Ni and $K_2Cr_7O_7$.

Figure 3. *In vitro* antagonism of the fungus *Colletotrichum gloeosporioides* in King's B culture medium. A - Isolate 291; B - Isolate 248; C - Isolate 287.

Seven isolates were able to show *in vitro* antagonism against the fungus *Alternaria alternata* for both culture media tested, and only isolate 290 showed no antagonism (in King's B culture medium), allowing the fungus to grow on its colony (Figure S2). In this test, the antagonism exhibited by this isolate was weak. There was no inhibition halo, and the bacterial isolates only prevented fungal growth over their colonies, suggesting possible competition for nutrients.

In the antagonism test for the fungus *Colletotrichum gloeosporioides*, isolates 248, 291, and 287 showed halo formation, being efficient on the fungus in King's B medium (Figure 3). For the BDA medium, seven isolates exhibited weak antagonism, showing only the growth of both

microorganisms, and only isolate 290 in BDA medium showed no antagonism, allowing the growth of the fungus on its colony.

The fungus *Phyllosticta citricarpa* was inhibited by all isolates, except for isolate 290, which presented weak antagonism. Isolates 248, 263, 287, 320, 274, 309 and 291 were able to produce halos around the fungal colony (Figure S3).

The pathogen *Geotrichum citri-aurantii* was inhibited by only two isolates, 309 and 287, which were able to exhibit halos around the fungal colony in King's B medium (Figure S4 - A/B). The remaining isolates 274, 320, 263, 248, and 291 presented weak antagonism, suggesting only nutritional competition, and isolate 291 did not present antagonism to *Geotrichum citri-aurantii* in King's B medium, with the fungus covering its colony (Figure S4 - C).

Production of siderophores

The production of siderophores was verified by cultivating isolates in Petri dishes in an iron-deficient culture medium. The results show that of the 8 isolates of the same lineage, only 6 were able to develop under iron deficiency without inhibiting their growth (Figure S5). Through the presence of a yellow halo around the colony, it is possible to identify the positive reaction for siderophores by the CAS method.

The time for production of halos around colonies was different for each isolate, with halos between 8 and 15 days after inoculation. Isolates 290 and 291 did not develop as well as the others, with weak orange halos around their colonies.

Discussion

Through *in vitro* tests for the production of enzymes of biotechnological interest, with eight *B. subtilis* strains, it was possible to verify that all strains were able to solubilize inorganic phosphate and produce amylase and cellulase (Figure S1, a, b, c). *In vitro* tests are essential to understand and know the abilities of microorganisms (Rocha et al., 2017).

Phosphate solubilization observed in all strains indicates that such isolates provide the availability of this element for plants to promote their growth, improving their performance, according to previous studies (Lobo, 2018; Diaz 2018; Santos et al., 2020).

Plants can absorb P in only two soluble ionic forms, monobasic and dibasic (Glass, 1989; Bhattacharyya and Jha & Saraf, 2012), as this nutrient is the most limiting, after N. Phosphorus is an indispensable and essential macronutrient for plant growth and production, acting on important processes such as energy transfer and storage, photosynthesis, cell division, root growth, respiration, seed formation, as well as fruit and grain quality (Khan et al., 2009). In the soil, it is possible to find large reserves of total phosphorus; however, the amount available to plants is minimal, since most of this element is found in insoluble forms, reflecting the impossibility of plants to absorb it (Rodríguez, 1999; Freitas et al., 1997).

Plant growth-promoting bacteria of the genus *Bacillus* can solubilize phosphate through the production of phosphatase enzymes and the release of organic acids that are excreted in the soil (Hariprasad et al., 2009; Patiño-Torres et al., 2014). Such substances modify the organic and inorganic forms of unavailable phosphate into assimilable chemical forms, which can be stored once incorporated into the root, and when necessary, phosphorus can be taken to the upper part of the plant, being accessible for metabolic reactions of plants (Alexander, 1978; Kpomblekou-A and Tabatabai, 1994; Jha & Saraf, 2015). Previous studies carried out with *Bacillus* for maize and cotton (Lobo, 2018; Diaz, 2018) presented results for efficient phosphorus solubilization, showing significant concentrations in the dry matter of roots and shoots.

All *Bacillus* strains were able to degrade starch. Starch is a carbohydrate synthesized by plants and is a high-molecular-weight glucose polymer that can accumulate as a type of energy reserve for most higher plants and is essential for many microorganisms (Van Der Veen et al., 2000; Vieille & Zeikus, 2001). In most plants, starch results from the storage of accumulated sugars, which can be distinguished in two forms: branched amylopectin and nonbranched amylose. Amylopectin and amylose accumulate in the form of insoluble starch grains inside amyloplasts and chloroplasts of plant cells, which can be fragmented into monosaccharides through hydrolysis reactions (Amaral et al., 2007; Evert & Eichhorn, 2014). Studies carried out by Santos et al. (2020) found that these strains promoted the growth and development of maize plants compared to the control, which suggests a direct symbiosis with plants, as such strains were able to provide essential elements for their development and plants possibly supplied the microorganism with this carbon source in the form of energy, favoring its stability.

All *Bacillus* strains were able to degrade carboxymethylcellulase. Bacteria of the genus *Bacillus* tend to produce cellulases in the presence of CMC, but this does not always occur when they are exposed to other substrates (Ito, 1998; Rastogi et al., 2010). The production of cellulase by endophytic microorganisms is seen as a mechanism to assist colonization of the host plant. This type of penetration is active and independent of natural openings or wounds that occur during plant growth. *Bacillus* penetration can trigger systemic host resistance, which is known as another mechanism of action of biological control agents (Hallmann et al., 1997).

Bacillus strains were capable of resisting the six metals tested - Cu, Zn, Cd, Cr, Ni and $K_2Cr_7O_7$, with IC_{50} values above 0.5, except for strains 248, 263 and 320 for Cd (Figure 2). Isolates that can be grown in high concentrations of trace elements depend on their resistance capacity. Resistance to such contaminants for some bacterial species could be developed by characteristics of their metabolism and genome through a variety of resistance systems mediated by plasmids, chromosomes and transposons. Although resistance to metals can occur due to prolonged exposure, which benefits the selection and multiplication of stress-tolerant microorganisms caused by metals, some resistance mechanisms can occur through active transport, reduction of the sensitivity of cellular targets to metal ions, exclusion by permeable barriers and intra- and extracellular sequestration (Hutchinson and Symington, 1997; Bruins, et al., 2000; Lopes et al., 2011).

Of the eight *Bacillus* strains, three - 274, 290 and 291 - were resistant to Cu at a concentration of 2.5 mmol L^{-1} (Figure 1-a); two - 248 and 320 - were resistant at a concentration of 2.0 mmol L^{-1}; two - 287 and 309 - were resistant at a concentration of 1.5 mmol L^{-1}; and only one – 263 - showed the highest resistance at 3.0 mmol L^{-1}. Copper is an essential micronutrient for prokaryotic and eukaryotic cells; however, at high concentrations, copper can cause enzymatic

inactivation, blockage of biochemical reactions, cell lysis, interference in the process of gene translation and transcription and enzymatic inactivation (Nies, 1999; Matyar, et al., 2010; Orell, et al., 2010).

Regarding resistance presented to Zn, five strains - 248, 274, 287, 290 and 309 - were able to develop at a concentration of 5.0 mmol L^{-1}, two strains - 263 and 290 - were resistant at a concentration of 3.0 mmol L^{-1}, and only one – 309 - was resistant at a concentration of 1.5-2.5 mmol L^{-1} (Figure 1-b). Ali et al. (2009) reported the resistance of *Bacillus subtilis* at a Zn concentration of 10.43 mM. Zinc is an essential element for microbial metabolism, constituting an important component for many enzymes and binding proteins, and is essential for cell performance; however, at high concentrations, zinc can become toxic, forming complexes (Bruins et al., 2000; Dopson et al., 2003).

Of the eight *Bacillus* strains, only six were resistant to Cd^{+2}, presenting a drop at a concentration of 1.5 mmol L^{-1} (Figure 1-d). Ayano et al. (2014) found that a cadmium concentration of 2.0 mmol L^{-1} inhibited the growth of bacteria isolated from the soil. The presence of this element can cause irreversible damage to bacterial cells, since cadmium binds to respiratory proteins, producing reactive oxygen, leading to a decrease in cell density, reducing the growth rate and even causing death (Lee et al., 2001; Ma et al., 2009).

When analyzing resistance to Cr, this element is known to be able to be present in several different oxidation states, with its oxidation state possibly ranging from -2 to +6. However, only Cr(III) and Cr(VI) are stable forms in nature, with Cr(VI) typically in two forms, chromate (CrO_4^{-2}) and dichromate ($Cr_2O_7^{-2}$), due to the solution pH (Shen and Wang, 1994; Dermou et al., 2007). When using Cr(III) - $CrCl3 \cdot 6H_2O$, strains 290 and 291 were observed to present higher resistance, developing at 5 mmol L^{-1}. For strain 274, the concentration that most interferes with development was 4.5 mmol L^{-1} (Figure 1-e), with the other strains interfering at a concentration of 3.5 mmol L^{-1}. When strains were submitted to Cr(VI)-$K_2Cr_7O_7$, seven isolates showed the same resistance behavior to the metal, with concentration values of 2.0 mmol L^{-1}, and only one strain (274) was able to develop at a concentration of 2.5 mmol L^{-1} (Figure 1-c). The result of such resistance occurred in a medium containing Cr, which may refer to the use of Cr(III), a less toxic state of this metal, for the preparation of the culture medium. Although this metal presents toxicity in trivalent and hexavalent forms, some bacteria were observed to be tolerant to this element. Resistance to metals is related to the production of chelating substances that can bind and complex metals, enabling the development of such microorganisms in ecosystems where microbiota are in constant competition (Ullah et al., 2015).

To determine the resistance of all *Bacillus* strains to trace element Ni, a concentration of 2.5 mmol L^{-1} (Figure 1-f) was sufficient to hinder their development. Although nickel is an important metal for the activity and constitution of metalloenzymes (Marschner, 1995; Negi et al., 2014), it can cause changes in cytosine methylation patterns causing DNA hypermethylation or hypomethylation, which can cause susceptibility of chromosomes to breakage or chromosomal instability (Kovalchuk et al., 2001). The interaction of this metal with microorganisms can lead to induced genotoxicity, where Ni can interact with chromatin proteins and form protein-DNA cross-links (Rossman, 1995; Anjum et al., 2015).

Resistance to toxic metals is a determining factor to be pointed out in studies on the remediation of contaminated areas, which is occasionally linked to the capacity for survival, growth and bacterial development, given the high concentrations of trace elements (Kang et al., 2016).

The activity exerted by bacilli against pathogens was demonstrated for the fungi *Alternaria alternata, Colletotrichum gloeosporioides, Phyllosticta citricarpa* and *Geotrichum citriaurantii*, which can cause diseases in orange orchards (Table 2). The action of bacteria occurs through *in vitro* fungal growth inhibition, in which all strains were able to present antagonism, except for strain 290, which showed no antagonism for the fungus *Alternaria Alternata* in King's B medium, *Colletotrichum gloeosporioides* in BDA medium, and *Geotrichum citri-aurantii* in King's B medium. *Bacillus* is a genus of bacteria that has antagonistic power of greater relevance, standing out for its ability to form endospores resistant to adverse circumstances and to present an abundance of antagonistic mechanisms to inhibit and reduce the defenses of phytopathogens, which allows its species to survive in specific ecological niches (Lanna Filho et al., 2010). In addition, bacteria of the genus *Bacillus* have the characteristic of producing hydrolytic enzymes such as cellulase, which degrade components of the cell wall of other microorganisms, giving the bacteria of the genus *Bacillus* the characteristic of mycoparasitism (Zago et al., 2000). Chen et al. (2008) found that *Bacillus subtilis* was able to produce 14 volatile antifungal compounds that inhibited the development of *Botrytis cinerea*, which causes gray mold in vegetables and fruits. Braga Junior et al. (2017) tested seven *Bacillus subtilis* strains against *Fusarium subglutinans, Curvularia luneta* and *Bipolaris* spp. and found that the isolates demonstrated potential in biological control and were effective in inhibiting mycelial growth.

The production of siderophores occurred after 8 days of inoculation, and only 6 strains were able to form an orange halo around the colony (Figure S5). Isolates 290 and 291 did not show significant growth, presenting weak halos around the colony.

Siderophores are highly electronegative secondary metabolites whose production is a characteristic that provides advantages to the survival of microorganisms in the most different environments, since the collaboration of iron in essential biological methods is a significant factor of microbial competition (Lynck et al., 2001). Siderophores are low molecular weight compounds that act in the capture of iron in the environment due to their high affinity for Fe^{+3}, where, although Fe^{+3} presents low solubility, siderophores are able to act as chelators of this cation and transport it to the interior of cells (Hider and Kong, 2010). Plant growth can be promoted indirectly through the production of siderophores, as well as biological mechanisms, which can prevent the proliferation of phytopathogens around the roots due to the sequestration of rhizospheric iron (Buysens et al., 1996; Davison, 1998).

Materials and Methods

The *Bacillus subtilis* strains used in this work belong to the collection of microorganisms from the laboratory of Soil Microbiology, Department of Microbiology of the "Júlio de Mesquita Filho" State University of São Paulo, FCAV/UNESP - Jaboticabal/SP.

Enzyme Degradation Capacity

The enzymatic degradation capacity was evaluated to identify the biotechnological potential that *B. subtilis*

isolates present for the production of phosphatase, amylase and cellulase.

Phosphatase

Biochemical tests were performed with *B. subtilis* isolates to identify the production of phosphatase using solid NBRIP culture medium (Nautiyal et al., 1999): 20 g of glucose; 5 g of Ca_5 $(OH)(PO_4)_3$; 5 g of $MgCl_2 \cdot 6H_2O$; 0.25 g of $MgSO_4 \cdot 7H_2O$; 0.2 g of KCl; 0.1 g of $(NH_4)_2SO_4$; 1.5% agar and pH 7.0 for a total volume equal to 1 L. Plates were incubated for 72 hours in B.O.D. The detection of the calcium phosphate solubilization capacity by bacteria was observed by the appearance of a clear halo around the colony.

Amylase

The production of amylase was evaluated using starch-agar medium: K_2HPO (0.3 g/L); $MgSO_4 \cdot 7H_2O$ (1.0 g/L); NaCl (0.5 g/L); $NaNO_3$ (1.0 g/L); starch (10 g/L); pH 6.9. Plates were submitted to B.O.D. at 30 °C for five days. After cultivation, plates were stained with Lugol (1% I2: 2% KI - potassium iodide) for 15 minutes, discarded and washed with 0.85% saline solution for 10 minutes, followed by a final discard. The appearance of a clear halo around the culture demonstrated the presence of amylase (Souza et al., 2008).

Cellulase

The production of cellulase was evaluated using BHB medium supplemented with 0.5% carboxy-methyl-cellulose (CMC), which was submitted to B.O.D. at 30°C for five days. After culture, the detection of the cellulase enzyme was evaluated by the appearance of a clear and yellowish halo around the culture after staining plates with 0.1% Congo red in water (Souza et al., 2008). All tests were performed in triplicate.

Test of resistance capacity to trace elements

The ability to resist trace elements was verified before the development of cell growth in solid medium containing different cadmium, chromium, nickel, zinc, copper and potassium dichromate concentrations. For this test, isolates were previously grown in nutrient broth (3 g of meat extract; 5 g of meat peptone; 1 g of NaCl for 1 L, pH 7.0) and incubated in B.O.D. at 28°C for 24 h. After this period, isolates were inoculated in plates containing nutrient agar medium (5 g of peptone; 3 g of yeast extract; 1 g of NaCl; 9 g of agar; for 1 L, pH 7.0) (Rodrigues et al., 1986) with cadmium, chromium, nickel, zinc, copper and potassium dichromate ions at the following concentrations: 0.1; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 4.0 and 5.0 mmol L^{-1}, obtained from solutions prepared by dissolving $CdCl_2 \cdot H_2O$, $CrCl_3 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $K_2Cr_7O_7$ in deionized water.

Statistical analysis of the resistance capacity to trace elements

The results of variables of the resistance capacity to trace elements were submitted to the survival analyses mathematical model, of sensory analyses of resistance or survival, the resistance or survival function was also used for the Weibull distribution to describe the effect of survival in different trace element concentrations through equation 1.

$$F_{(t)} = 1 - e^{-\left(\frac{t}{\beta}\right)^{\alpha}}$$

where β is the scale parameter and α is the shape parameter.

Data were analyzed using Statistic 10 software (StatSoft, Tulsa, USA) and R software for statistical computing, version 3.2.4 (CORE_TEAM, 2017).

In vitro antagonism against phytopathogenic fungi

B. subtilis isolates were evaluated for the potential of brown spot control in citrus varieties caused by *Alternaria alternata*, citrus floral rot caused by *Colletotrichum gloeosporioides*, black citrus spot caused by *Phyllosticta citricarpa*, and sour rot caused by *Geotrichum citriaurantii*. Cultures were kindly provided by Ph.D. Prof. Kátia Cristina Kupper. The cultures belong to the collection of microorganisms of the Laboratory of Phytopathology and Biological Control of the "Sylvio Moreira" Advanced Citrus Research Center/IAC, Cordeirópolis/SP, Brazil.

A culture medium disc containing mycelial growth of the fungus was transferred to the center of Petri dishes containing King's B medium and in BDA medium in duplicate. Petri dishes were kept at 30°C for 48 hours. Bacteria were kept growing for 24 h in King's B medium at 30°C and then transferred at 4 points equidistant from each other and from the center of Petri dishes. Subsequently, Petri dishes were submitted to incubation under the same conditions again for 24 and 48 h. Antagonism could be observed by the formation of inhibition halos of the fungus by bacteria.

Production of siderophores

The production of siderophores was evaluated in solid medium MM9 (Schwyn and Neilands, 1987) supplemented with a solution containing chrome azurol S (CAS), Fe^{3+} and hexadecyl trimethyl ammonium bromide (HDTMA), as described by Louden et al. (2011).

Conclusions

The analyses carried out did not allow a better understanding of the mechanisms involved in each isolate; however, it was possible to identify strains with the capacity to degrade enzymes, showing resistance to metals, production of siderophores, and antagonistic action. These results open the way to clarify which genetic characteristics are involved in each mechanism of action so that isolates can be used as new bioinoculants in bioremediation processes and as biotechnological products.

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