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# Endophytic bacteria isolated from both healthy and diseased *Agave sisalana* plants are able to control the bole rot disease

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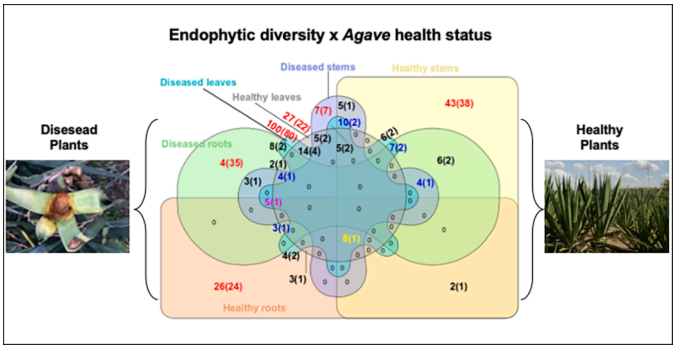
## HIGHLIGHTS

- Diseased plants tend to show higher population densities of bacterial endophytes.
- No association was found between plant health status and bacterial diversity.
- Less than 2% of the endophytes showed direct antagonism against the pathogen *in vitro*.
- The efficacy of the biocontrol agents is independent of the health status of the host plant.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Sisal is an economically and socially significant perennial crop for the semiarid region of the world. Bole rot disease, caused by black aspergilli, mainly by *Aspergillus welwitschiae*, is responsible for great losses due to the ability of the pathogen to kill the plant. The health status of plants that harbor endophytic biocontrol agents (BCAs) is being investigated in this study. We conducted experiments with endophytic bacteria from different parts of healthy and diseased sisal plants to select potential BCAs to control the disease. Studies on the populational densities showed that leaves have less bacteria when compared to roots and stems, and that diseased plants tended to have higher populations of bacterial endophytes. These results were obtained with both conventional plate counting and qPCR. A total of 497 isolates were screened as potential biocontrol agents on sisal discs and nine were selected as having direct antagonistic activity. Five isolates were from healthy and four from diseased plant parts. All these nine isolates significantly decreased the bole rot disease incidence in two field experiments and were identified by sequencing of the 16S rDNA as species belonging in the genera *Bacillus*,

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*Brevibacterium*, *Burkholderia* (2 isolates), *Paenibacillus*, *Pseudomonas* and *Serratia* (3 isolates). Isolate 466 of *Burkholderia* lowered 80% of disease incidence in both field experiments, which was not significantly different from the non-inoculated, negative control. We discussed these findings under an ecological-physiological interaction standpoint, as well as the possibilities of including diseased plants in bioprospection strategies and applying the selected isolates in the development of bioproducts.

## 1. Introduction

In the last decades biological control is gaining importance in agriculture worldwide due to its higher safety towards the environment, livestock and human health when compared to agrochemicals. One of the most frequent sources of biocontrol agents (BCAs) against specific pathogens are natural populations of rhizobacteria or endophytes associated with plants that suffer from the corresponding disease (Yuan et al., 2017; Purahong et al., 2018). According to the autochthony concept, organisms from the same origin tend to provide better performances in biological control, as the putative BCAs display higher adaptability to the target pathogen's niche (Fravel, 2005; Pomella et al., 2007). However, this hypothesis has not been investigated in detail. Furthermore, there are many studies showing that allochthonous microorganisms perform as well in biocontrol (Li et al., 2013; Cavero et al., 2015; Cruz-Magalhães et al., 2017).

*Agave sisalana* (sisal) leaves are the source of natural hard fibers used to manufacture twines, cordage for maritime usage, handcrafts and composites/parts for the construction and automobile industries (Nava-Cruz et al., 2015; FAO, 2019). Leaf residues that result from fiber extraction may be used as fertilizers, animal feed, raw material for biofuels and also a source of pharmaceutical compounds such as hecogenin, which is an important raw material for the synthesis of steroidal drugs (Silva et al., 2008; Debnath et al., 2010; Escamilla-Treviño, 2012; FAO, 2019). This species is well adapted to semiarid regions and is cultivated in Brazil, Tanzania, Kenia, Madagascar, China and Mexico (Silva et al., 2008; Sharma and Varshney, 2012; FAO, 2019). Semiarid and arid environments occupy approximately 40% of the land surface (Khresat et al., 2004), with over 30% of human population living in these regions, which are among the poorest and most marginalized people on Earth (WWAP, 2009). The Brazilian semiarid region, known as the Caatinga biome, roughly corresponds to 10% of the country's territory (IBGE SIDRA, 2015). This biome shows irregular precipitations and average temperatures as high as 32 °C in some areas (INMET, 2018). Sisal is one of the few plant species economically exploited in the Caatinga biome and is traditionally produced by small farmers that utilize familiar labor (Silva et al., 2008).

Sisal production is affected by an array of biotic and abiotic stressing factors, but the bole rot disease, caused by several species of black aspergilli, is the most significant stressing agent in Brazil and several other countries (Kimaro et al., 1994; Santos et al., 2014). The causative agents of this disease include *Aspergillus welwitschiae*, *A. brasiliensis* and *A. tubingenisis* (Santos et al., 2014; Duarte et al., 2018). The symptoms of the disease are internal red lesions in the stem (or bole) and yellowing of the aerial parts that ultimately lead to plant death (Santos et al., 2014). Therefore, finding strategies to successfully manage the disease with environmentally friendly approaches is of great interest.

There is a widespread assumption that microorganisms from healthy plants have more chances of becoming BCAs able of mitigating the disease when compared to those from diseased plants (Fravel, 2005). Healthy plants are indeed most commonly exploited as sources of commercially useful antagonists (Raza et al., 2009; Yang et al., 2012; Hazarika et al., 2019). There are few studies addressing the influence of the health status of plants as sources of prospective BCAs (Huang et al., 2013). In this sense, some studies have shown that the process of infection by phytopathogens can stimulate the plant to produce different compounds that can be secreted by the roots, which may contribute to the selection of specific colonizers and thus influence the composition, activity and recruitment of microbial populations in the rhizospheric/endospheric environment (Rudrappa et al., 2008; Compant et al., 2010; Trivedi et al., 2012; Huang et al., 2013). In this study, we adopted an approach based on culturable bacterial endophytes from different parts of healthy and diseased sisal plants. The study of culturable microorganisms provides less information on taxonomic diversity when compared to culture-independent methods; however, isolation and characterization of culturable endophytes is a relevant strategy not only to obtain information on the structure and ecological functions of these communities, but also to prospect isolates with potential to control the disease and serve as the basis for bioproducts.

Considering all the relevant aspects involved in the understudied sisal × *Aspergillus* spp. pathosystem, the objectives of this study were (i) to investigate the influence of the health status of sisal plants on the population densities of the endophytic community associated with different tissues; (ii) to verify the impact of health status on the diversity of culturable endophytic bacteria isolated from different parts of sisal plants; and (iii) to investigate if microbes from healthy or diseased plants (Supplementary Fig. 1) differ in their ability to control bole rot disease. To accomplish these tasks, we performed isolation, quantification and characterization of the genetic diversity of culturable endophytic bacterial community from healthy and diseased plants of *A. sisalana*, with emphasis on the identification of strains with potential for further development as biological control agents of bole rot disease.

## 2. Materials and methods

### 2.1. Quantification of culturable endophytic bacteria in healthy and diseased plants

Bacterial endophytes were isolated from six healthy and six diseased 7-month-old sisal plants collected in the municipality of Barrocas (Bahia, Brazil; 11° 31′ 50.1″ S, 39° 04′ 28.9″ W), located in the Caatinga biome. In the laboratory, these plants were separated into roots, stems and leaves, and approximately 2 g of each plant part was separately processed for isolation. After surface-disinfestation with 70% ethanol (3 min), NaOCl 1% (5 min), and three washes in sterile distilled water, samples were macerated in a sterile mortar with a pestle. Grinded tissues were then serially diluted and plated in triplicates onto Petri dishes containing TSA (5 g peptone, 15 g tryptone, 5 g NaCl and 15 g agar per litre). Plates were incubated for 72 h at 25 ± 2 °C. Controls were prepared for each sample by plating the water from the last wash. Only the plates/dilutions that did not show any bacterial growth in the respective control plates were used for further experiments. Isolated colonies were randomly selected and preserved in glycerol 20% at −80 °C until use. A completely randomized design with a factorial scheme of 2 symptomatological states (healthy and diseased) × 3 plant parts (roots, stems and leaves), with six replicates per treatment were adopted. For the statistical analysis, the data were transformed into $\log_{10}$CFU. The statistical analyses were done through the R platform, with mean comparisons done by the *t*-test for the same tissue (roots, stems or leaves) in healthy and diseased plants, or by the Tukey's test among different tissues for healthy or diseased plants; both tests were performed at 5% probability level.

### 2.2. Quantification of endophytic bacteria in health and diseased plants by qPCR

To quantify the endophytic bacterial population in sisal plants by real time PCR, healthy and diseased plantlets were collected in the municipality of Jacobina, Bahia (11° 10′ 50″ S, 40° 31′ 06″ W). For DNA extraction, only samples of the bole part of the plant were used, because it tends to show higher culturable populations of bacteria and it is the plant tissue directly affected by the disease. Samples of 0.1 g of the stem tissue were surface-disinfested and grinded in liquid nitrogen. The material was transferred to 1.5 ml tubes containing 720 ml of pre-heated extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl and 1.25% SDS), homogenized and then incubated at 65 °C for 15 min. Proteins were removed by precipitation after adding 225 μl of 5 M potassium acetate, vortexing and incubating for 20 min at −20 °C. Samples were then centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to a clean tube. The DNA was precipitated by adding 2/3 of the volume with cold isopropanol and the remaining inhibitors were removed by washing the DNA with 300 μl of 70% cold ethanol. The DNA was resuspended in 50 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at −20 °C until use. Four groups were defined to be quantified by qPCR in this study: Eubacteria, which refer to the total populations of bacteria; Enterobacteriaceae, *Pseudomonas* spp. and *Bacillus* spp., each using the appropriate primers described in Supplementary Table 1. Standard curves were built using PCR products from each set of primers. After amplification, the products were quantified using the Qubit® 2.0 Fluorometer. Afterwards, serial dilutions were performed and PCR was used to determine the value of each cycle threshold (Ct). The Ct values were compared to the logarithm of the concentration or the number of copies in each sample. For these amplifications, the reactions conditions for each primer pair were the same as those described in Supplementary Table 1.

### 2.3. DNA fingerprinting analysis by BOX-PCR

A total of 348 randomly selected isolates of endophytic bacteria from roots, stems and leaves of healthy and diseased sisal plants were used for genetic diversity studies with BOX-PCR. A rapid genomic DNA extraction procedure was used for the isolates grown on TSA medium for 24 h at 25 °C. A loopful of each isolate was transferred to 1-ml microfuge tubes containing 100 μl of extraction buffer (0.05 M NaOH + 0.25% SDS) and incubated at 97 °C under agitation (800 rpm) for 15 min. Tubes were cooled to room temperature for 2 min and centrifuged at 10,000 rpm for 1 min. The DNA in the supernatant was diluted 20X in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and stored at −20 °C until use.

DNA samples from each isolate were amplified using the primer BOX A1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic et al., 1994). The amplification reactions contained 2.5 μl of 10x PCR buffer, 1.5 μl of 10 mM dNTPs, 2 μl of 50 mM $MgCl_2$, 2.5 μl of primer at 10 μM, 0.3 μl of *Taq* DNA polymerase at 5 $U.\mu l^{-1}$, 3 μl of template DNA at 30 $ng.\mu l^{-1}$, and ultra-pure water up to a final reaction volume of 25 μl. The PCRs were performed with an initial denaturation step at 94 °C for 7 min, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 53 °C, and 8 min extension at 65 °C, and a final extension step at 65 °C for 15 min. After the amplifications, 3 μl of loading dye (0.25% bromophenol blue and 60% glycerol in ultrapure water) were added to each 25-μl sample reaction. These samples were applied on 2% agarose gel containing EtBr (0.5 $\mu g.ml^{-1}$), with a 100-bp DNA ladder as size standard, and run for 5 h at 50 V in TAE buffer (90 mM Tris-Acetate and 1 mM EDTA). The patterns of amplicons generated by the BOX-PCR were converted into a binary data matrix (1 for presence and 0 for absence of a particular size band in the gel) and was used to calculate the Jaccard's coefficient of similarity. The software Past 2.1 (Hammer et al., 2001) was used for cluster analysis with the Neighbor-Joining method. The reliability of the dendrogram was tested through bootstrap analysis with 1,000 resampling. The dendrogram was visualized and edited with the software Mega 5.0 (Tamura et al., 2011).

### 2.4. Selection of antagonistic bacteria against the pathogen on sisal stem discs

A total of 497 bacterial isolates were used to perform bioassays on sisal stem discs, 348 used in the BOX-PCR experiments (see above), plus 149 additional isolates obtained in a second isolation phase of this study. Cell suspensions of these isolates were prepared by growing them for 20 h in ST broth (17 g tryptone, 3 g peptone, 5 g NaCl, 2.5 g $K_2HPO_4$, 2.5 g glucose per litre), centrifuging for 3 min at 10,000 rpm, washing the pellet once and re-suspending it in sterile distilled water. Each bacterial suspension was adjusted in a spectrophotometer to an optical density of 0.05 at a 550-nm wavelength. The fungal pathogen, *Aspergillus welwitschiae* isolate 131, was obtained from a diseased sisal plant collected at the municipality of Barrocas (Bahia). Spore suspensions of the fungus were obtained by scraping spores from 10-day-old cultures grown on PDA medium (potato, dextrose, agar) at 25 °C. All working spore suspensions were adjusted to the same amount of conidia (see below).

Bioassays for the selection of endophytic bacteria were done on sisal stem discs measuring 1 cm of diameter and 0.5 cm of thickness, which were obtained from healthy sisal plants using a sterilized standard cylindrical borer. Stem discs were surface-disinfested with ethanol and NaOCl as described above and individually placed on the surface of a sterile moistened filter paper accommodated at the bottom of plastic boxes (with lids) of 100 ml volume. Five discs per bacterial sample were individually inoculated with 0.1 ml of a cell suspension and after 3 h with 0.1 ml of *A. welwitschiae* spore suspension adjusted to $10^7$ conidia $ml^{-1}$. Plastic boxes were placed at 28 ± 2 °C for 5 days until the evaluation. The experiment was assessed using a diagrammatic scale with decreasing grades (from strong to weak effects) ranging from 1 to 4, where 1 = no mycelial growth or sporulation (strongest effect); 2 = only mycelial growth; 3 = sporulation on half of the disc; 4 = sporulation on the whole disc (Supplementary Fig. 2). The experiment was installed in a complete randomized design with the 497 endophytic isolates as treatments, a negative control with sterilized water only and a positive control with *A. welwitschiae* only. Each treatment had five replicates.

### 2.5. Identification of selected bacterial isolates and screening for selected antibiotics

Among the 497 tested bacterial isolates, nine were selected as potential biocontrol agents; these selected isolates were preliminarily identified by sequencing of the 16S rRNA gene. The PCR primers used in this study were: 8 FN (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1429r (5′ GGTTACCTTGTTACGACTT 3′) (Turner et al., 1999). PCR was done in 25-μl reactions containing 2.5 μl 10x PCR buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl), 2.0 μl of dNTPs at 2.5 mM each, 0.75 μl of each primer at 10 $pmol.\mu l^{-1}$, 2.0 μl of 50 mM $MgCl_2$, 0.4 μl of Taq DNA polymerase (5 $U.\mu l^{-1}$), 3 μl of DNA at 10 $ng.\mu l^{-1}$, with final volume completed with ultrapure sterilized water. The amplification conditions included an initial denaturation at 94 °C for 2 min, followed by nine cycles at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min, plus 29 cycles at 94 °C for 45 s, 48 °C for 45 s and 72 °C for 1 min; and a final extension step at 72 °C for 5 min. Sequencing was performed by the Sanger method in an ABI Prism™ 310 Genetic Analyser using the amplification primers and the BigDye® Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's recommendations (Applied Biosystems). Sequences were assembled with Sequencher 5.0 (Gene Codes). The newly obtained 16S rRNA sequences were deposited in GenBank (see accession numbers in Table 3) and were compared with others in the rDNA/ITS database using the BlastN program (Altschul et al., 1997).

The DNA of the nine bacterial isolates was used in PCR reactions using the specific primers and the amplification conditions described in the references contained in Table 1. These samples were applied on 1% agarose gels containing EtBr (0.5 $\mu g.ml^{-1}$) with the 100-bp DNA ladder

**Table 1**
Primers used in the amplification of genes involved in the biosynthesis of antibiotics.

| Target gene | Primers | Sequences | References | Isolates with positive amplification |
|---|---|---|---|---|
| *phzFA* | PHZ1 | GGCGACATGGTCAACGG | Delaney et al., 2001 | – |
| (Phenazine) | PHZ2 | CGGCTGGCGGCGTATTC | | |
| *prnD* | PRND1 | GGGGCGGGCCGTGGTGATGGA | De Souza and Raaijmakers, 2003 | 127 and 130 |
| (Pyrrolnitrin) | PRN2 | YCCCGCSGCCTGYCTGGTCTG | | |
| *phlD* | B2BF | ACCCACCGCAGCATCGTTTATGAGC | McSpadden et al., 2011 | – |
| (2,4-DAPG) | BPR4 | CCGCCGGTATGGAAGATGAAAAAGTC | | |
| *bamC* (Bacillomycin) | BACC1F | GAAGGACACGGCAGAGAGTC | Ramarathnam et al., 2007 | – |
| | BACC1R | CGCTGATGACTGTTCATGCT | | |
| *zmaR* (Zwittermicin A) | 678 | ATGTGCACTTGTATGGGCAG | Raffel et al., 1996 | 512 |
| | 667 | TAAAGCTCGTCCCTCTTCAG | | |

as the size standard. The presence of bands with specific sizes for each primer pair was used as indicative of the antibiotic under study.

### 2.6. Biological control of bole rot disease in the field

Field experiments were performed at the municipality of Ourolândia (Bahia, Brazil), located in the semiarid region (10° 57′ 29.7″ S, 41° 04′ 25.5″ W). Sisal plantlets with ~ 20-cm height were planted in plastic polyethylene bags containing non-sterilized soil and grown for 45 days under field conditions. The plantlets were wounded on the stem with two needles fixed to a wooden base. The needles were 2-cm long, 0.5-cm apart and with a diameter of 0.5 mm. Aliquots of 1 ml of bacterial suspensions prepared as described above for screening on sisal discs were applied on these injuries, and 3 h later 1 ml of fungal spore suspension ($10^7$ conidia.ml$^{-1}$) was applied on them. The treatments were (i) the nine bacterial isolates previously selected in the bioassays with sisal stem discs, (ii) a positive control with *Aspergillus welwitschiae* only, and (iii) a negative control with water only. The experiments were installed in a complete randomized design with 11 treatments and 15 replicates per treatment. After 30 days, the disease incidence was recorded by observing external and internal symptoms. Typical bole rot symptoms include yellow leaves and rotten stems; affected stem tissues may acquire colors varying from light brown to dark red. Non-parametric chi-square of Wald with contrasts was performed with the SAS software (SAS, 2000). The experiment was done twice and analysed separately.

## 3. Results

### 3.1. Bacterial populations tend to be higher in tissues of diseased plants

The endophytic bacterial populations in different parts from healthy and diseased sisal plants were quantified by plating serial dilutions of ground surface sterilized tissues. Population densities of culturable endophytic bacteria ranged from $10^4$ to $10^6$ CFU.g$^{-1}$ of dry tissue and were on average 1.6x higher in diseased as compared with healthy plants (Fig. 1). Densities of endophytic bacteria in diseased sisal stems were higher than in leaves of both healthy and diseased plants and roots of diseased plants; these densities were not significantly different between roots and stems of healthy plants (Fig. 1A). Higher populations

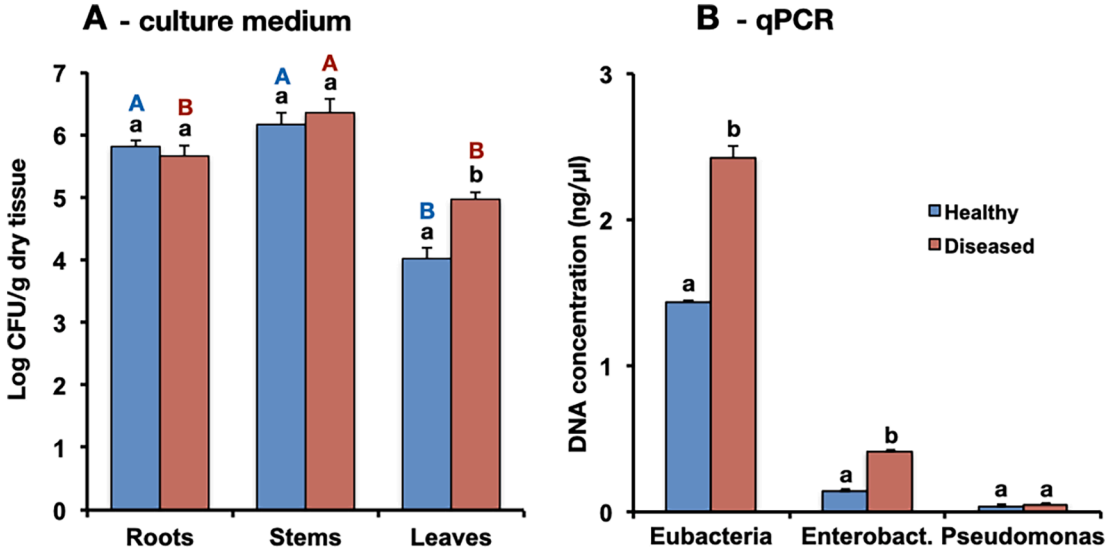


**Fig. 1.** Population densities of endophytic bacteria in tissues of healthy and diseased sisal plants. (A) Population densities of culturable endophytic bacteria in healthy and diseased dry tissues of sisal plants as determined by dilution plating on culture medium. Samples of the different tissues were surface sterilized and grinded, and serial dilutions were plated on TSA medium to determine the population densities in different plant tissues. Mean comparisons should consider (i) uppercase letters of the same color (blue letters for different tissues of healthy plants and red letters for tissues of diseased plants), or (ii) lowercase letters for healthy and diseased plants within each tissue type. Means followed by the same letter are not significantly different according to Tukey's test ($P > 0.05$) for uppercase, colored letters, or according to the *t* test ($P > 0.05$) for lowercase letters. Means of six replicates are shown and the error bars represent the standard error of the means. (B) Bacterial population densities in healthy and diseased stems of sisal plants determined by qPCR with primers for total bacteria (Eubacteria), the family Enterobacteriaceae, and the genus *Pseudomonas*. DNA concentrations in samples from healthy and diseased stem tissues were determined by qPCR. Data are means of three replicates. Means followed by the same letter are not significantly different according to the *t*-test ($P > 0.05$). Error bars represent the standard error of the means. The color legends for the bars (red and blue) are the same for both (A) and (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were also found in diseased leaves as compared with healthy ones ($P < 0.05$), whereas roots and stems showed similar bacterial densities for both healthy and diseased plants (Fig. 1A). Similar trends (i.e., higher bacterial population densities in diseased as compared with healthy sisal plants) were also observed for real time PCR for the Eubacteria- and *Enterobacteriaceae*-specific primers (Fig. 1B). On the other hand, populations of the genus *Pseudomonas* were lower than the two other groups and also did not significantly differ between healthy and diseased plants. Densities of *Bacillus* populations could not be determined with standard curves, but they have shown to be the lowest among the evaluated taxa based on the Ct method (Supplementary Table 2).

### 3.2. Diverse communities are found in both healthy and diseased plants

A BOX-PCR analysis was employed in 348 culturable endophytic isolates obtained from the first round of bacterial isolation from the sisal tissues in order to verify the overall level of microbial diversity in the healthy and diseased plants. The results indicated that the 348 isolates were clustered in 236 groups with a unique banding pattern (Fig. 2; Supplementary Fig. 3; Supplementary Table 3). From these, 246 isolates (70.7%) representing 206 BOX groups (87.3%) occurred exclusively in single tissues; the remaining isolates were classified as follows: 61 isolates in 21 BOX groups co-occurred in two tissues, 28 isolates in 7 BOX groups co-occurred in 3 tissues, 5 and 8 isolates in a single BOX group each co-occurred in 4 and 5 sisal tissues, respectively (Fig. 2; Supplementary Table 3). The number of BOX groups within each plant part was not significantly different according to the chi-square test ($P > 0.05$) (Supplementary Table 3). The isolates/BOX groups that occurred exclusively in single sisal tissues were the only ones showing identifiable and comparable patterns of occurrence (Fig. 2; Supplementary Tables 3 and 4). Accordingly, the percentages of exclusive BOX groups per single sisal tissue were similar when diseased and healthy tissues were compared, i.e. 86% of the single-tissue BOX groups occurred in healthy and 84% in diseased tissues. Although the number of isolates differed among tissues, the average percentage of BOX groups that occur exclusively in one sisal tissue in relation to the total number of isolates tended to be higher in roots and stems (89% and 88% of the BOX groups on average, respectively) as compared to leaves (79% of the BOX groups) (Supplementary Table 4). These results indicate that there was more diversity in roots and stems than in sisal leaves.

### 3.3. Screening and identification of the most promising isolates against the bole rot pathogens

The 348 isolates used in the BOX-PCR studies, plus 149 other isolates from healthy sisal plants obtained in a second phase of this research (not shown) were tested in screening experiments against the bole rot pathogen. Approximately 98% of the isolates received the grades 2, 3 or 4 in the *in vivo* screening experiments, indicating a weak direct antagonistic effect against *A. welwitschiae* on sisal stem discs (Table 2). The number of isolates from healthy or diseased plants was not associated with the scale grade in the stem disc test according to the chi-square test ($P > 0.05$) (Table 2). Among the nine isolates with the lowest grade (1 = strongest antagonistic effect), five were from healthy and four were from diseased plant parts, thereby indicating a lack of relationship between the health status of the plant and the direct antagonistic potential of the corresponding isolates.

These nine most promising isolates were identified by sequencing of their 16S rRNA gene. The results indicated they belonged in the genera *Brevibacterium*, *Bacillus*, *Burkholderia* (2), *Paenibacillus*, *Pseudomonas* and

**Table 2**
Biocontrol assay on sisal stem discs to test the potential of endophytic bacteria against *Aspergillus welwitschiae*.

| Scale grade* | Healthy plants | | | Diseased plants | | | Total |
|---|---|---|---|---|---|---|---|
| | Roots | Stems | Leaves | Roots | Stems | Leaves | |
| **1** | 1 | 4 | 0 | 1 | 0 | 3 | **9** |
| **2** | 3 | 2 | 6 | 5 | 1 | 12 | **29** |
| **3** | 24 | 43 | 43 | 15 | 11 | 61 | **197** |
| **4** | 34 | 46 | 40 | 41 | 15 | 86 | **262** |
| **Total** | **62** | **95** | **89** | **62** | **27** | **162** | **497** |

The 497 bacterial isolates are distributed according to the grade attributed to them with the scale shown in supplementary Fig. 2*, the health status of the plants and the plant part they were obtained from. Sisal stem discs were sprayed with each bacterial suspension, 3 h later with *A. welwitschiae* and evaluated 5 days later. Chi-square (P = 0.05) was used to test the association between the number of BOX groups in each part of diseased and healthy plants and grades of the scale. No significant differences were found.

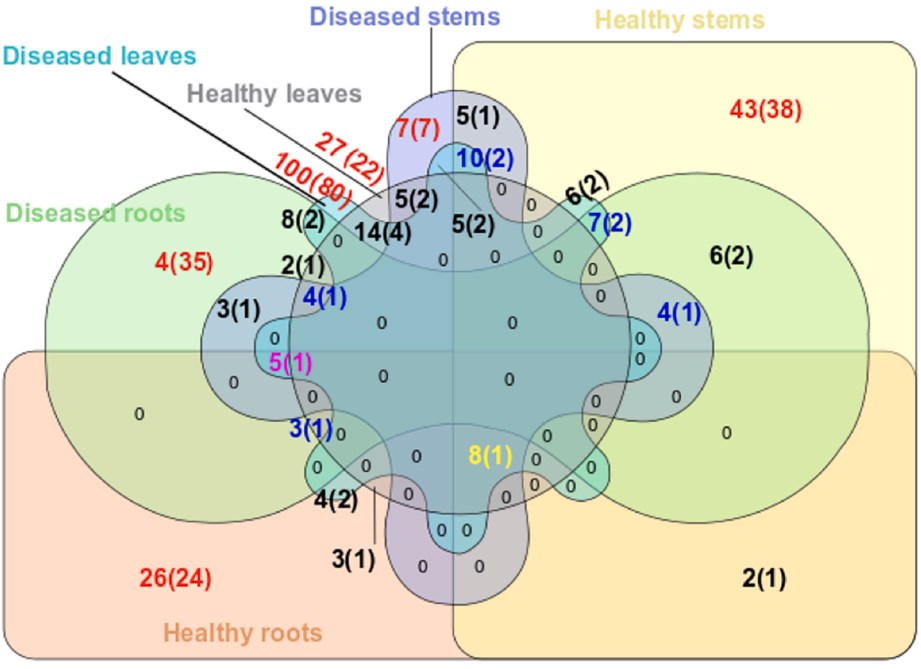


**Fig. 2.** Venn diagram for the 348 endophytic bacterial isolates with their distribution in different healthy and diseased parts of sisal plants. The numbers between parenthesis show the distribution of the 236 groups defined by the BOX-PCR. Red numbers indicate the isolates and BOX groups that occurred exclusively in a single plant tissue; black numbers indicate isolates/BOX groups in two plant tissues; blue in three; pink in four and yellow in five sisal tissues. More information on the isolates and their origin is presented in Supplementary Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

*Serratia* (3), although their taxonomic species could not be determined (Table 3). The identities of their sequences with their closest match in the public rRNA curated databases varied from 99.3 to 99.9% (Table 3).

These isolates with more direct antagonistic potential to control the bole rot disease were tested with specific primers for the presence of genes responsible for the synthesis of five different antibiotics (Table 1). Isolates 130 and 127 of *Serratia* were shown to harbor genes for pyrrolnitrin biosynthesis and *Paenibacillus* 512 possessed genes for zwittermicin biosynthesis, indicating that these three isolates are able to produce the above-mentioned antibiotics.

### 3.4. Biocontrol activity of the selected isolates against the bole rot disease in the field

The nine isolates selected in the screening experiments (see above) were tested against the bole rot disease in two independent field experiments (Fig. 3). In both experiments, all bacterial isolates tested significantly decreased disease incidence and differed from the positive control inoculated with *A. welwitschiae*, which showed the highest disease incidence (Supplementary Table 5). The inhibition of disease incidence in the field ranged from 33 to 80% in the first experiment and from 25 to 83% in the second experiment. *Serratia* 127 and *Burkholderia* 466 provided the highest reductions in disease incidence in both experiments, but *Burkholderia* 466 was the only isolate that did not significantly differ from the non-inoculated (negative) control in both experiments (Fig. 3). Thus, there was no correlation between the health status of the source of biocontrol agents and their activity in the field.

## 4. Discussion

Understanding the interactive roles of plant-associated microorganisms in both natural and agricultural environments is a requirement to obtain novel sources of microbes with potential application in the biocontrol of plant diseases. Studies comparing quantitative and functional aspects of the microbiota of healthy and diseased plants are scarce, and mainly focused on the rhizosphere environment (e.g. Huang et al., 2013; Cao et al., 2016; Lee et al., 2017). An interesting idea that has been raised postulates that microorganisms selected from healthy plants exhibit better performances in promotion of growth and/or biocontrol of plant pathogens (Fravel, 2005; Costa et al., 2013). The underlying rationale is that healthy plants are better sources of antagonistic microorganisms, since the microbial population in such environment may have been selected to keep the pathogen in check. The main question addressed in this study was whether the health status of a plant host can ultimately determine the probability of finding active endophytes against a given pathogen, and thus, defining the activity/-potential of their associated bacterial endophytes as antagonists for use in biological control. Bole rot disease caused by black aspergilli is the main disease of sisal (*Agave sisalana*), an economically and socially significant perennial crop for the semiarid region of the world, as it is traditionally produced by small farmers that utilize familiar labor. This pathosystem has been systematically studied by our research group (Santos et al., 2014; Cruz-Magalhães et al., 2017; Barbosa et al., 2018), and was used here as a case study. By investigating populational densities, diversity and biocontrol activity of endophytic bacteria obtained from healthy and diseased plants, our results suggested that diseased plants tend to show higher population of bacterial endophytes than healthy ones; however, the genetic diversity and the proportion/activity of potential BCAs were not influenced by the health status of the host plant.

Studies have shown that a healthy host plant is able to control the densities and the diversity of the microbiota growing on its surface or inside its tissues (Badri and Vivanco, 2009; Bisseling et al., 2009; Zamioudis and Pieterse, 2012). This control of microbial colonization ultimately reflects the expression of the induced systemic resistance (ISR) system, which acts by limiting the growth of pathogens and endophytes in the same manner. All microbes in healthy plants are initially recognized as invaders by the plant, but after such a limitation in their colonization, the systemic antimicrobial activity decreases to the pre-infection levels (Zamioudis and Pieterse, 2012). On the contrary, a diseased plant tends to lose its capacity to limit the growth of microorganisms, including endophytic microbes that may act deleteriously, despite being initially beneficial. It has been shown that endophytic colonization of *Trichoderma* elicits ISR in cucumber plants and as a result cell wall reinforcements and hydrolytic enzymes are overproduced, preventing the invasion of the plant vascular tissue by this beneficial microbe (Yedidia et al., 1999). Based on this view, the higher densities of bacterial endophytes found in diseased sisal plants (Fig. 1) as compared to the healthy ones may be explained by a generally lower capacity of these plants to limit the growth of invading/associated microorganisms (Zamioudis and Pieterse, 2012). As discussed above, the higher population densities observed for diseased plant tissues, however, does not necessarily imply a higher genetic diversity and/or biocontrol activity of the endophytic community, as seen in our case (Figs. 2 and 3). Nevertheless, the lack of differences between healthy and diseased plants can also be considered relevant, as it demonstrated that healthy is not the only valid status for a plant to deserve prospective studies towards finding microbial antagonists (see further discussion below).

Concerning the genetic diversity of endophytic bacteria, some studies have found this parameter as being higher in diseased plants when compared with healthy ones (Reiter et al., 2002; Yang et al., 2012; Purahong et al., 2018). This is in contrast to our results, which showed that the diversity of endophytes was independent from the health status of sisal plants (Fig. 2; Supplementary Fig. 3 and Supplementary Table 3). These contrasting results may be explained by the isolation/assessment method we used, which was based on analyses of the culturable bacterial communities from different plant parts. Additionally, in other pathosystems, i.e. citrus huanglongbing (HLB; (Trivedi et al., 2010, 2012), a decreased microbial diversity associated with diseased plant roots was observed with 16S gene libraries and real time PCR. However, further efforts are needed to investigate the densities of the endophytic

**Table 3**
Identification of selected endophytic bacteria from sisal by 16S rDNA gene sequencing.

| Isolates | Identification in this study | Isolation source | Accession number [a] | Fragment size (bp) | Closest match in databases/ accession number [b] | Identity (%) |
|---|---|---|---|---|---|---|
| 90 | *Brevibacterium* sp. | Healthy stem | KU207994 | 1,381 | *Brevibacterium sediminis*$^T$ KX356313 | 99.3 |
| 105 | *Bacillus* sp. | Healthy root | KU207995 | 1,373 | *B. pumilus*$^T$ NR_112637/ *B. safensis*$^T$ NR_113945 | 99.9 |
| 127 | *Serratia* sp. | Diseased leaf | KU207993 | 1,025 | *S. rubidaea*$^T$ NR_114232 | 99.3 |
| 512 | *Paenibacillus* sp. | Diseased leaf | KF922668 | 1,412 | *P. bovis*$^T$ NR_148889 | 99.6 |
| 130 | *Serratia* sp. | Diseased leaf | MH561725 | 1,348 | *S. rubidaea*$^T$ NR_114232 | 99.3 |
| 469 | *Serratia* sp. | Healthy stem | MH561726 | 1,088 | *S. rubidaea*$^T$ NR_114232 | 99.5 |
| 466 | *Burkholderia* sp. | Healthy stem | MH561727 | 1,391 | *B. contaminans*$^T$ JX986975/ *B. metallica*$^T$ AM747632 | 99.9 |
| 475 | *Burkholderia* sp. | Healthy stem | MH561728 | 1,428 | *B. contaminans*$^T$ JX986975/ *B. metallica*$^T$ AM747632 | 99.9 |
| 248 | *Pseudomonas* sp. | Diseased root | MH561729 | 702 | *P. montellii*$^T$ AB681966 | 99.7 |

[a] Accession numbers obtained in this study.
[b] Curated rRNA/ITS databases with type strains ($^T$) was used.

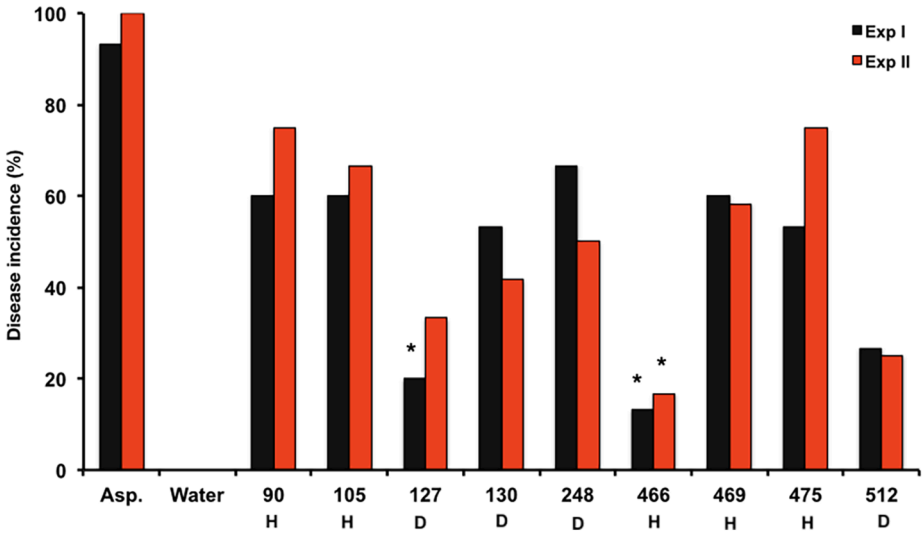


**Fig. 3.** Biological control of bole rot disease with selected endophytic bacteria in two independent field experiments. All treatments with bacterial isolates, including the negative control significantly differed from the positive control inoculated only with the pathogen, *A. welwitschiae*, according to Wald's contrast test at 5% probability (Supplementary Table 5). Asterisks indicate the experiments in which the respective isolates did not significantly differ from the negative control (with absence of the pathogen). The letters H and D underneath the isolate identification indicate the status of their source, i.e. whether healthy or diseased plant tissue.

community. Culture-independent, molecular approaches are expected to provide at least three to four times more operational taxonomic units (Rastogi et al., 2013; Santos et al., 2019), which can reveal a higher diversity that can be possibly overlooked by the culturable-dependent studies. Nevertheless, even employing high-throughput sequencing methods, other studies have also confirmed the lack of association between diversity and the health status of the plants (e.g. Lee et al., 2017). The levels of microbial diversity in plants under biotic stresses seems to be a species-specific characteristic (Purahong et al., 2018). In terms of distinct plant tissues, our study showed a higher diversity in roots and stems of sisal plants than in leaves (Fig. 2; Supplementary Table 3). These results were not unexpected, since endophytes are considered to be a subset of the rhizospheric community, frequently showing a different composition (Compant et al., 2010; Lundberg et al., 2012; Leite et al., 2013; Bulgarelli et al., 2013; Coleman-Derr et al., 2016).

There are no studies on the characterization and evaluation of the biocontrol activity in a comparative manner between endophytic bacteria from healthy and diseased sisal plants. To our knowledge, this is the first study to explore this question in depth, not only by employing a quantitative comparative approach with a large number of culturable endophytic bacteria from sisal, but also by simultaneously addressing diversity and antagonism in the same set of isolates. From our *in vitro* screening experiments, only ~ 1.8% of the 497 tested isolates showed potential as BCAs, a proportion within the range observed for other systems (Chen et al., 1996; De Souza et al., 2016; Guetskyl et al., 2002). Moreover, as seen in Fig. 3, and similarly to the diversity results mentioned above, no association between the antagonistic potential of selected isolates and the health status of the plants were observed, as also described in other systems (Reiter et al., 2002; Huang et al., 2013; Lee et al., 2017). At this point, two other possibilities cannot be discarded: (i) a screening on a larger number of culturable isolates, from a larger number of source plants might yield differences in antagonistic activity of associated microbes between diseased and healthy plants (see Reiter et al., 2002), and (ii) the use of culture-dependent methods may never be able to detect such differences, as only a small portion of the true microbial diversity can be assessed in such way, and culturing procedures may select specific groups of the bacterial communities (see Reiter et al., 2002; Purahong et al., 2018).

The mechanism of biocontrol that we focused in this study was direct antagonism against the bole rot pathogen, *A. welwitschiae*. The nine most promising biocontrol isolates were also screened for the presence of genes responsible for the production of five different antibiotics previously described as being involved in the biocontrol of plant pathogens (Table 1); only three of these isolates appeared to produce the antibiotics pyrrolnitrin and zwittermicin (Table 1), which suggests that either there might be other types of antibiotics involved in this control for the remaining six isolates, and/or the direct antagonistic mechanism may be of a different nature. Further studies are warranted to elucidate the mechanisms of action of these isolates against this pathogen. It is also worth noting that, although *in vitro* tests of direct antagonism is a widely used methodology, various studies have shown that the *in vitro* preliminary results do not always correlate positively with biocontrol activity under greenhouse and/or field conditions (e.g. Rajkumar et al., 2005; Ran et al., 2005; Lemessa and Zeller, 2007; De Souza et al., 2016). In this sense, it is possible that certain bacteria may be weak antibiotic producers, but strong colonizers of the rhizosphere. Rhizospheric colonization (i) is a trait associated with antagonists that exhibit good biocontrol performance, (ii) is a requirement to promote growth in the field and greenhouse (Compant et al., 2010; Ren et al., 2012; Huang et al., 2013; Han et al., 2016), and (iii) is the gateway to the interior of plant tissues (Compant et al., 2010). By comparing the effect of rhizospheric bacteria from diseased and healthy tomato plants on the control of bacterial wilt (*Ralstonia solonacearum*), Huang et al. (2013) showed that the biocontrol efficacy and growth promotion phenotypes correlated positively with the isolates' ability to colonize the roots and negatively with the results of direct antagonism. Therefore, the ability to produce antibiotics may not always be a characteristic that guarantees the success of a biocontrol agent under field conditions.

The bacterial endophytes selected for antagonistic activity from the *in vivo* and field experiments were preliminary identified as belonging to Firmicutes, Proteobacteria and Actinomycetes, which are among the most common phyla encountered inside plants (Bulgarelli et al., 2013). Lined-up experiments to be carried out in a near future will include inoculation of these selected endophytes (excluding the potential human pathogens; see below) in micropropagated sisal plants, in order to verify their potential as growth promoters as well. Obviously, culturable isolates bearing more than one beneficial effect tend to be preferable for the development of bioproducts. In addition, these experiments will also include the application of these isolates in different combinations as it has been suggested that the use of beneficial microbial consortia can increase efficacy, consistency and reliability of bioproducts under a variety of environmental settings (Stockwell et al., 2011; Purahong et al., 2018).

The isolates of endophytic *Burkholderia* and *Serratia* selected in this study were among the best biocontrol agents of sisal bole rot disease. The initial identification we performed based on 16S sequencing indicated that the *Burkholderia* isolates belong in the *B. cepacia* species complex and the *Serratia* isolates are closely related to *S. rubidaea*. Both

of these bacterial groups were reported as effective biocontrol agents in other systems, as plant pathogens and also as opportunistic human pathogens associated with cystic fibrosis patients (Abd-Alla et al., 2011; Ansari et al., 2019; Magalhães et al., 2017; Parke and Gurian-Sherman, 2001; Soenens and Imperial, 2020; Ursua et al., 1996; Zhang et al., 2005). Several attempts have been made along the years by numerous authors to discriminate between beneficial and pathogenic isolates, including metabolic/physiological profiling, such as production of pectinase, bacteriocins and antibiotic sensitivity; genetic/molecular taxonomic approaches, including the *Burkholderia cepacia* epidemic marker (BCESM), ribotyping, RAPD, RFLP, MLST, genome sequencing, phylogenomics and presence of virulence factors; and phenotypic characterization such as pathogenicity to onion (Abreo and Altier, 2019; Parke, 2000; Parke and Gurian-Sherman, 2001; Seo and Tsuchiya, 2004; Trakulsomboon et al., 1997; Wallner et al., 2019). Unfortunately, none of these characters were sufficiently safe to distinguish between pathogens and beneficial environmental isolates (Parke, 2000; Parke and Gurian-Sherman, 2001; Eberl and Vandamme, 2016). Therefore, we do not recommend the use of these isolates as living biocontrol agents before they are unequivocally shown not to be pathogenic to humans. One option is the formulation of products containing bioactive compounds together with heat-killed cells of these bacteria to control plant diseases. Such products, although not biocontrol per se, have not encountered problems in registration and marketing (Marrone Bio Innovations, 2021).

In the same way that the idea of using autochthonous microbes for disease control is not necessarily better than allochthonous microorganisms (Magalhães et al., 2017), the search for good biocontrol agents in the endophytic community of diseased plants should also not be neglected as a bioprospection strategy. Taking the pathosystem *Arabidopsis thaliana-Pseudomonas syringae* as an example, phytopathogen infection caused an increase in malic acid levels in root exudates; such an increase has contributed to the recruitment of a *Bacillus subtilis* strain, which colonized the rhizoplane, induced the expression of resistance genes and, as a consequence, decreased the effects of the pathogen in the aerial part of the plant (Rudrappa et al., 2008). In this context, understanding the conceptual framework of the holobiont/hologenome view, which states about the close functional interaction among microbes and host cells, can help explain the complexity of these relationships (Bordenstein and Theis, 2015; Catania et al., 2017).

It is well documented that microorganisms can play an important role in alleviating the abiotic stress of many agricultural crops (Grover et al., 2011; Choudhary et al., 2016; Vardharajula et al., 2017). In this sense, it is interesting to explore unique traits that may help in the development of methods to improve agricultural production (Dey et al., 2018). Sisal is considered a strategic plant species under the perspective of global climate changes, since it has several traits that can be explored in semiarid regions and in temperate latitudes with dry environments (Yang et al., 2015; Rajaud and Noblet-Ducoudré, 2017). The present study added key information regarding the potential of associated endophytic microbes in providing useful biocontrol activity for a crucial disease in the sisal crop. Therefore, it is interesting to further study this plant's microbiome as a possible source of novel isolates/functions that may help in the adaptation of agricultural species to the foreseen abiotic stresses expected from global warming (Guevara-Avendaño et al., 2019). The authors also hope that further research in other systems to quantify and characterize the microbiota of both healthy and diseased plants should be pursued, as not only important comparative knowledge is to be revealed, but also because it will turn diseased plants as a relevant alternative source of potential BCAs.

## Author contributions

ACMS, AFJS and POS performed and planned the experiments; VCM, PSA, ACMS and JTS performed data analyses; ACMS, JTS, VCM, PASM and LLL conceived the study, wrote the manuscript and contributed with ideas and financing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2021.104575.

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