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Compatibility and combination of selected bacterial antagonists in the biocontrol of sisal bole rot disease

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Abstract Sisal (*Agave sisalana* Perrine ex Engelm; Asparagaceae) bole rot is a devastating disease caused by *Aspergillus niger* Tiegh, which contributes to the decline of this crop in Brazil. Currently, there are no control measures available, but biocontrol is being investigated as a promising management strategy. Five previously selected bacterial isolates were identified by 16S rRNA sequencing as *Paenibacillus* sp. 512, *Brevibacterium* sp. 90 and *Bacillus* sp. 105, BMH and INV, and tested for their capacity to control bole rot in vitro and in field experiments. Individual bacterial isolates and their combinations significantly inhibited mycelial growth and spore germination of *A. niger*. In two independent field experiments, the application of isolates 512, 105, 90, INV, and 127 + INV reduced disease incidence to levels varying from 44 to 75%. Although there was no synergistic effect in their combined use, these bacteria have potential to be used against bole rot disease in the field.

Keywords *Agave sisalana* (Asparagaceae) · *Aspergillus niger* · *Bacillus* sp. · *Paenibacillus* sp. · Disease incidence and severity

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Introduction

Sisal (*Agave sisalana* Perrine ex Engelm) is an herbaceous monocotyledonous species that belongs in the Asparagaceae family. Although originated in Mexico's Yucatan peninsula, sisal is well adapted to the semiarid regions of countries such as Brazil, China, Tanzania and Kenia (Silva et al. 2008). The Brazilian semiarid region is known as Caatinga and occupies 9.9% of the Brazilian territory (IBGE 2015). It is characterized by long drought periods with irregular precipitation that varies from 300 to 800 mm year^{-1}, high evapotranspiration and annual mean temperatures as high as 32 °C in some areas (CPRM 2006; INMET 2015).

The leaves of sisal are the major source of a hard fibre that is utilised to make twines, cordage, kraft

paper, handicrafts, and as composites in the construction and automobile industries (Silva et al. 2008). Brazil is the main producer and exporter of sisal fibre in the world since 1961 with the production 138,008 tons in an area of 156,536 ha in 2014 (FAOSTAT 2017). Most of the sisal cultivation in the Brazilian semiarid region is done by small farmers that employ familiar labor. It is one of the few crops that may be grown successfully in the Caatinga biome and is a source of income to approximately 600,000 people (Silva et al. 2008).

Sisal is considered to be a rustic plant with few problems with pests and diseases, but in Brazil and in Tanzania it is attacked by a disease known as bole rot, caused by black aspergilli (Coutinho et al. 2006; Kimaro et al. 1994; Santos et al. 2014). *Aspergillus tubingensis* Mosseray, *A. brasiliensis* Varga et al. and species in the *A. niger* Thieg. complex have been identified as the cause of bole rot (Kimaro et al. 1994; Santos et al. 2014). Diseased plants present internal lesions in the bole or stem, which exhibit a red colour upon exposure. Leaves of these plants are not suitable for fibre extraction and the plants die as the disease progresses (Coutinho et al. 2006; Fig. 1).

In spite the importance of this pathogen on sisal cultivation and the socio-economic significance of this crop, scientific studies on sisal in general and more specifically on the control of bole rot have historically been neglected. However, recent studies conducted by our research group suggested that beneficial bacteria might be used to control bole rot disease (Silva 2012; Magalhães et al. 2017). In this study, previously isolated bacterial agents, which had been preliminarily selected based on in vitro tests, were first identified, tested alone or in combinations in laboratory and field experiments in order to control the sisal bole rot disease.

Materials and methods

Microorganisms used in this study

The bacterial isolates used in this study were deposited in the collection of microorganisms maintained by the Microbiology Laboratory of Recôncavo da Bahia Federal University (UFRB), Brazil. The bacterial isolates were endophytic and obtained from leaves, bole or roots of healthy sisal plants and from soil collected close to healthy plants at the municipality of Barrocas in the sisal-growing region of Bahia, Brazil. Isolations were done by surface sterilizing fragments of approximately 5 mm^2 of sisal plant parts by immersing in 70% alcohol for 1 min, then in 1% NaOCl for 2 min followed by three washes in distilled sterile water. These plant fragments were plated on nutrient agar (NA) and incubated at 25 °C for 48 h. Soil was diluted in 10× steps and 100 µl aliquots were spread on NA plates and incubated as described previously. The bacterial isolates obtained in this way were stored in 40% glicerol at − 20 °C for further studies. The isolates pre-selected as potential biocontrol agents against *Aspergillus niger* in a previous study (Silva 2012) are listed in Table 1. To prepare the cell suspensions, all bacterial isolates were grown on NA for 24 h at 28 °C and then transferred to nutrient

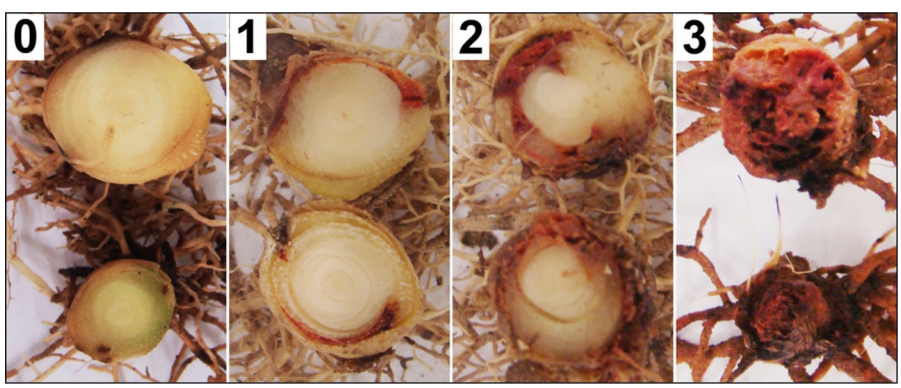

Fig. 1 Disease index used to score the severity of bole rot disease. Sisal plantlets were inoculated with spores of *A. niger* and 30 days later split open to observe the internal symptoms in the bole. Severity is scored as **0** no symptoms are visible; **1** red symptoms on the external leaves; **2** advanced symptoms in the internal part of the bole; **3** dead plant. (Color figure online)

Table 1 Identity of the bacterial isolates used in this study as determined by sequencing of the 16S rRNA gene

Isolate	Identification	Accession number	Source
90	*Brevibacterium* sp.	KU207994	Sisal bole
105	*Bacillus* sp.	KU207995	Sisal root
512	*Paenibacillus* sp.	KF922668	Sisal leaf
127	*Serratia* sp.	KU207993	Sisal leaf
INV	*Bacillus* sp.	KU207997	Soil
BMH	*Bacillus* sp.	KU207996	Soil

broth (meat extract 3 g and peptone 5 g l^{-1}) with shaking at 120 rpm at 28 °C for 12 h. Cell suspensions were adjusted to $A_{600} = 0.5$ using a spectrophotometer. These bacterial isolates were identified by sequencing a fragment of the 16S rRNA gene as previously described (Leite et al. 2013) and the accession numbers are deposited in GenBank (Table 1).

Aspergillus niger 131, deposited in the collection of microorganisms maintained by the Microbiology Laboratory of UFRB, was used in all experiments. Isolate 131 was obtained from a diseased sisal plant in the municipality of Conceição do Coité, Bahia. This isolate was identified by sequence analyses of fragments of the beta-tubulin (KU207998) and calmodulin (KU207999) genes. The fungus was routinely grown on PDA at 28 °C and spore suspensions were prepared by scraping the spores from eight-day-old plates and adjusting the concentration to 10^5 conidia ml^{-1}.

Compatibility between bacterial isolates

To determine the compatibility between bacterial isolates, in vitro assays were performed according to Sundaramoorthy et al. (2012). Cell suspensions were prepared as described previously, spread on 1/10 strength Trypticase Soy Agar (TSA) and full strength NA plates in all possible combinations of two isolates. Every two isolates were spread in perpendicular lines in relation to each other. Plates were incubated at 28 °C for 72 h and then observed for inhibition zones. Compatible isolates were able to grow over each other and inhibition zones were present between incompatible combinations (Fig. 2). Four replicates per treatment were adopted and the experiment was done twice for each culture medium.

(a)

	Bacterial isolates					
	512	105	90	127	BMH	INV
512		+	-	+	-	-
105	+		+	+	-	-
90	-	+		+	-	-
127	+	+	+		+	+
BMH	-	-	-	+		-
INV	-	-	-	+	-	

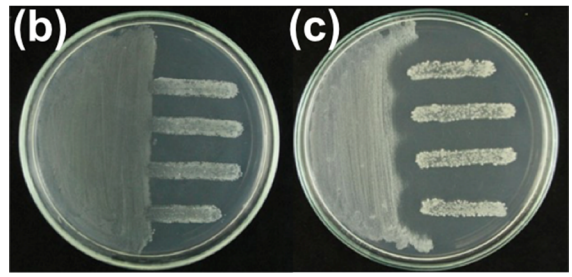

Fig. 2 Compatibility between bacterial antagonists in agar plates. **a** The plus signal indicates a compatible combination and a minus indicates an incompatible combination. **b** Compatible and **c** incompatible combinations on plates

Effect of bacterial isolates on *Aspergillus* spore germination and mycelial growth

The effect of bacterial isolates on *Aspergillus* spore germination was tested in experiments done in ELISA plates by adding either 100 µl of *A. niger* spore suspension plus 100 µl of the bacterial cell suspensions or 100 µl of a mixture containing equal amounts of bacterial suspensions of the combined isolates followed by the addition of 50 µl of PDB (Potato Dextrose Broth). Spore suspensions contained 10^5 conidia ml^{-1} and bacterial cell suspensions were adjusted to an $OD_{600} = 0.5$ as described previously. The treatments included six individual bacterial isolates, seven compatible combinations and controls comprised the liquid medium and sterile distilled water with 100 µl of *A. niger* spore suspension. Each treatment was repeated four times and plates were sealed and incubated at 37 °C for 16 h. After incubation, all treatments received one drop of lactophenol blue to stop fungal growth. The number of germinated spores in a total of 100 randomly selected spores was evaluated under a microscope. Spores with the length of germ tubes equal or superior to the double of the spore diameter were considered germinated. The

experiment was done twice in a completely randomized design with four replicates per treatment.

The effect of the bacterial isolates on mycelial growth of *A. niger* was determined by the culture pairing technique. Aliquots of 6 µl of the bacterial cell suspensions described previously were added to 5 mm-diameter paper discs. To test a combination, 6 µl of a mixture containing equal amounts of each suspension of the combined isolates was used. Two paper discs were placed at 1 cm from the border of each 9-cm diameter Petri plate containing PDA. A 5-µl aliquot of *A. niger* spore suspension was placed at the centre of each Petri plate after placing the bacterial isolates or the control with distilled water. The treatments were the six bacterial isolates, seven compatible combinations and the control was the plate with the filter papers with 6 µl of distilled water. Plates were kept at 28 ± 2 °C for eight days and colony diameter was evaluated every 24 h while the diameter of the inhibition halo was evaluated at the eighth day. The treatments were repeated three times (three plates with two filter papers) and the whole experiment was done twice. Time and incubation temperature for both experiments were determined as optimal conditions in previous pilot experiments.

Field experiments

Field experiments were installed at Tiquara community (10°26′20″S and 40°39′39″W), 27 km from Campo Formoso municipality, Bahia, which is located in the semiarid region of Brazil. The first experiment was performed in June 2014, which corresponded to the rainy season in this region, with a mild climate predominating. The daily average temperature ranged from 16.6 to 29.6 °C (average 21.9 °C), 45.3% cloud coverage, and occasional rain (total of 27 mm during the experiment). The second experiment was conducted in October 2014, in the summer, with average temperature of 27.4 °C (19.4–34.5 °C), without rain or cloud coverage. The soil of the area is alkaline with a low concentration of nutrients (Supplementary Material Table S1). Before the application of the treatments, two-month old sisal plantlets measuring 25-cm high were wounded on the stem with two needles fixed to a wooden base. The needles were 2 cm long, 0.5 cm apart and 0.5 mm of diameter. Aliquots of 100 µl of the bacterial suspensions described above were applied on each wound or 100 µl of a mixture containing equal amounts of each suspension in combined treatments. Twenty-four hours later 100 µl of *A. niger* suspension containing 10^7 conidia ml^{-1} was applied on each wound. The experiments were installed in a randomized block design and were done twice. The treatments were the six bacterial isolates, seven compatible combinations, one combination of all isolates including the incompatible ones, and controls with *A. niger* only and the water control. Experiment 1 was installed in three blocks containing four replicates of five plants per treatment per block, totalling 20 plants per treatment per block and 60 plants per treatment in the whole experiment. Experiment 2 contained three blocks with three replicates of five plants per treatment per block and a total of 15 plants per treatment per block and 45 plants per treatment in the whole experiment. Plantlets were transplanted to plastic bags containing 2 l of soil collected from the top layer (20 cm) of the area where the experiments were installed. They were kept in the field and were irrigated as needed. Disease incidence was assessed weekly up to 30 days and these data were used to calculate the area under the disease progress curve (AUDPC) according to Campbell and Madden (1990). After 30 days the plantlets were collected, split open to evaluate bole rot severity with a disease index described by Sá (2009). The level were 0 = absence of symptoms; 1 = initial lesion, slightly larger than the wound done by the needles, rot at the base of the most external leaf; 2 = advanced symptoms, internal rot in the stem, plant still alive; 3 = dead plant, rotten stem (Fig. 1).

Data analysis

Disease indexes (DI) were transformed into percentages by using the equation $ID = \Sigma[(G \times F)/(N \times 3)] \times 100$, where G is the grade assigned to severity with the scale, F is the frequency of each grade, N the number of plants evaluated, 3 the maximum grade of the scale according to McKinney's index (Aguiar et al. 2013). All data were evaluated for normality and homogeneity of variance using Lilliefors' and Levene's tests, respectively. AUDPC data were subjected to the analysis of variance and Scott–Knott's tests ($P < 0.05$). Results from the different experimental seasons were analysed separately with Scott–Knott's tests ($P < 0.05$). To determine whether there was or not a synergistic interaction in bacterial

combinations, Limpel's formula as described by Richter (1987) was used: $Ee = X + Y - X \times Y/100$, where Ee is the expected additive value of the combined bacterial agents, X and Y are the levels of control provided by each individual agent. A synergism was determined to be present when the combination produces a value higher than Ee and when the value is lower there is antagonism in the combination. Treatments without variability such as controls with values equal to 0 or 100 in all replicates were excluded from the analyses. All statistical analyses were conducted using R v. 3.0.2 statistical package (R Core Team 2016).

Results

Identification and in vitro compatibility between bacterial isolates

The six bacterial isolates used in this study were identified by sequencing the 16S region of the rRNA. When the sequences obtained were compared to the ones deposited in public databases they were 99 or 100% identical to the genera to which they were assigned (Table 1). The isolates were identified at the genus level due to the lack of resolution of this gene to determine bacterial species.

Incompatible isolates showed inhibition halos regardless of the direction they were spread on the plates. Seven combinations were compatible while eight were incompatible. Isolate 127 of *Serratia* was the only isolate which was compatible with all the others (Fig. 2). Isolates 90, 512 and 105 were inhibited by BMH and INV that also inhibited each other. The same responses were observed on TSA and NA media and in another independent experiment. Only compatible combinations were tested in further experiments, except for one treatment that included all isolates in the field experiments.

Inhibition of spore germination and mycelial growth

All bacterial isolates applied either individually or in combination reduced *A. niger* spore germination when compared to the control ($F_{12,39} = 784.6$, $P < 0.001$; Table 2). Mycelial growth was also reduced in comparison to the control treatment ($F_{13,42} = 15.7$, $P < 0.001$). The combinations generally showed spore germination similar to the levels obtained for the most active isolate of the combination (Table 2). Although all isolates significantly decreased mycelial growth, only treatments 512, BMH, and 512 + 105 reduced growth between 43 and 50% (Table 2). With the combination of isolates, no increased effect was observed for the inhibition of both spore germination and mycelial growth. A repeated experiment showed similar results.

Control of bole rot disease under field conditions

Most isolates reduced the incidence of bole rot when applied individually or in combination as compared to the control without bacteria in both field experiments (experiment 1: $F_{13,126} = 15.734$, $P < 0.001$; experiment 2: $F_{13,84} = 4.727$, $P < 0.001$). There was an effect of the blocks in field experiment 1 ($F_{2,126} = 7.40$, $P < 0.001$), but not in experiment 2 ($F_{2,84} = 2.148$, $P = 0.123$). Average AUDPC was lower in the first experiment when compared to the second (experiment 1: $F_{14,135} = 27.867$, $P < 0.001$; experiment 2: $F_{14,90} = 8.929$, $P < 0.001$). In the first experiment, four individual isolates and one combination and in the second experiment, all six individual isolates and two combinations showed a relatively lower ($P < 0.05$) disease incidence than the other treatments (Fig. 3). Overall, treatment of sisal plantlets with individual bacterial isolates resulted in lower disease incidences and AUDPC values in both field experiments when compared to combined applications (Fig. 3). Disease incidence was on average 47.4% in the first and 54.4% in the second experiment for individual bacterial isolates and 65.2% in the first and 73.8% in the second experiment for combined bacterial isolates. AUDPC values were respectively 603.9 and 718.1 for the first and second experiments in individual bacterial treatments. These values were 743.2 and 1014 for the first and second experiments in combined bacterial isolates. In general, the best treatments in terms of reduction in disease incidence were the isolates applied individually, with exception of 127 and BMH ($P > 0.05$), and the only combination that consistently reduced disease incidence in both experiments was 127 + INV. The combination of all six isolates did not improve biocontrol efficacy (Fig. 3). There were significant differences in biocontrol efficacies in both field experiments (experiment 1:

Table 2 In vitro effects of bacterial agents alone or in combination on spore germination and mycelial growth of *A. niger*

Treatments	Inhibition of spore germination (%)	Mycelial growth (cm)
512	99.2 ± 0.48 b*	1.7 ± 0.00 a*
105	98.0 ± 0.91 b	2.8 ± 0.14 b
127	95.7 ± 0.48 b	2.8 ± 0.09 b
90	87.2 ± 0.95 a	2.6 ± 0.21 b
BMH	98.5 ± 0.29 b	1.9 ± 0.06 a
INV	97.5 ± 0.29 b	2.1 ± 0.09 a
512 + 105	99.2 ± 0.48 b	2.6 ± 0.03 b
512 + 127	95.2 ± 0.75 b	1.9 ± 0.10 a
90 + 105	96.7 ± 2.02 b	2.7 ± 0.00 b
90 + 127	97.5 ± 0.65 b	2.8 ± 0.08 b
105 + 127	95.5 ± 1.94 b	2.7 ± 0.07 b
127 + BMH	97.0 ± 0.00 b	2.9 ± 0.03 b
127 + INV	98.2 ± 0.63 b	2.5 ± 0.29 b
Aspergillus	0.00	3.4 ± 0.06 c

Measurements were performed eight days after the installation of the experiments

*Means followed by the same letter in the columns are not significantly different according to Scott–Knott's tests at 5% probability. Values are means of four replicates followed by SE

$F_{13,126} = 15.734$, $P < 0.001$; experiment 2: $F_{13,84} = 4.727$, $P < 0.001$). According to Limpel's criterion (Richter 1987), biocontrol efficacies higher than the expected value in combinations of two biocontrol agents indicate synergism. Except for the combination 127 + INV in the first experiment, there were no synergistic effects on the reduction of disease incidence by the combination of two bacterial isolates (Table 3).

Discussion

In many instances, the combination of biocontrol agents has been shown to result in higher levels of control than the application of one agent alone (Roberts et al. 2005; Stockwell et al. 2011; Zangoei et al. 2014). However, successful combinations of biocontrol agents require compatibility between them (De Boer et al. 2003). In this study, six bacterial biocontrol agents selected in previous studies were tested for compatibility and the compatible combinations were further studied to control the pathogen in vitro and bole rot disease in the field.

The inhibition of mycelial growth and spore germination in vitro shown by the bacterial isolates (Table 2) was used as an indicative of their activity in the field. In accordance with most systems (Fravel 1988; De Souza et al. 2016), the in vitro experiments reported in this study were poor predictors of field performance in the case of the combinations. Microorganisms behave differently in vitro and in soil environments, when they are interacting with a multitude of other microbes (Lambers et al. 2009; Bakker et al. 2014; Braga et al. 2016). These complex interactions among microorganisms are just beginning to be understood. Interactions bacteria–bacteria and fungi–bacteria can lead to three outcomes: beneficial, neutral or detrimental to one of the partners taken into consideration (Frey-Klett et al. 2011). There are studies showing that certain strains of *Bacillus* attach and colonize the hyphae of *A. niger* leading to either growth stimulation or mycelial lysis (Benoit et al. 2014). In mutual beneficial interactions there is a rewiring of gene expression and a decrease in the production of antimicrobial metabolites by the fungal and the bacterial partners (Benoit et al. 2014). On the other hand, in antagonistic interactions, the bacteria produce lytic enzymes and antifungal compounds that result in fungal control (Podile and Prakash 1996). The bacterial isolates used were selected against *A. niger* in previous studies and for this reason their single

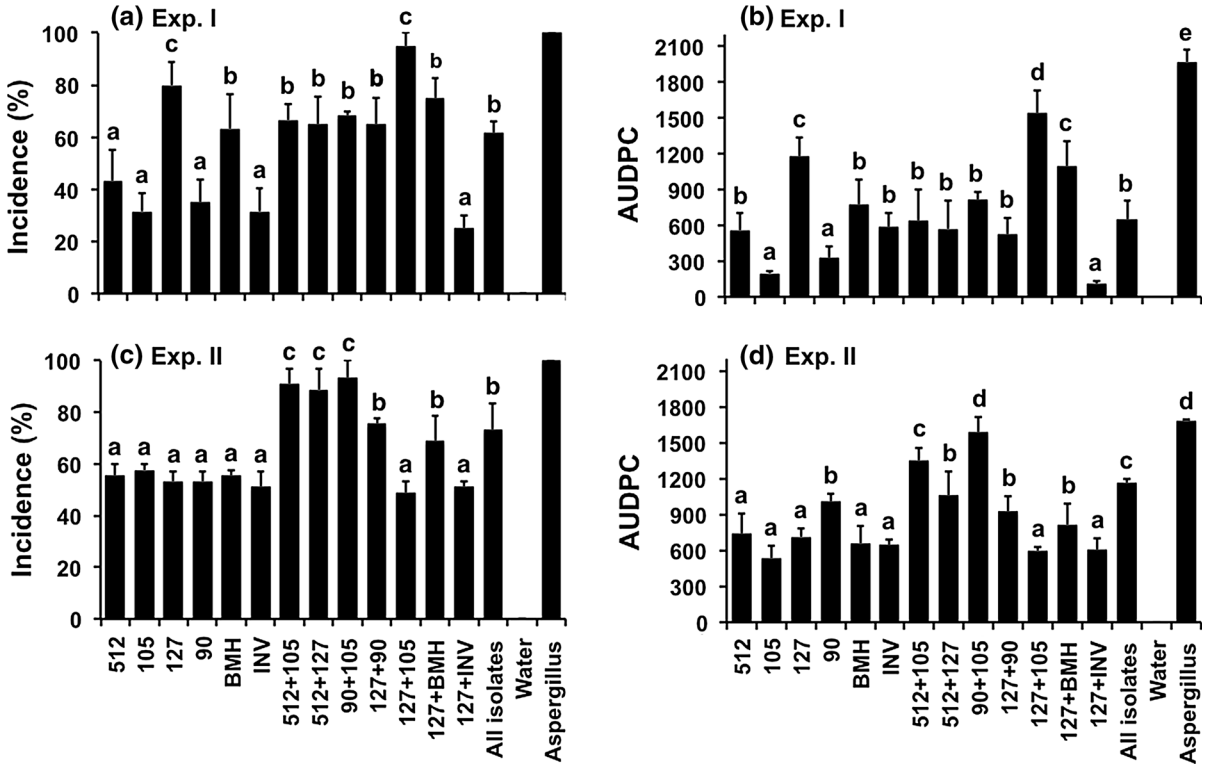

Fig. 3 Disease incidence and area under the disease progress curve (AUDPC) of sisal bole rot disease in two independent field experiments. **a**, **c** Incidence of bole rot disease in the field. **b**, **d** Area Under the Disease Progress Curve (AUDPC) of bole rot disease calculated with the severity data and the disease index showed in Fig. 1. Controls without variance were not included in the statistical analyses. Values are means of three replicates of 20 plants per replicate. Error bars represent SE. Means followed by the same letter are not significantly different according to Scott–Knott's tests at 5% probability. Statistical comparisons should be read for each graph separately

application always led to decreases in bole rot disease and also on mycelial growth and spore germination. Unfortunately, they were not selected as partners/mutual helpers or to compose a consortium of bacteria and therefore we had no effect of the combinations in most cases.

The fact that most isolates used in this study were identified as species of *Bacillus*, *Paenibacillus* and *Brevibacterium* may explain why more than 50% of the possible combinations between two isolates were incompatible. Gram-positive bacteria in these genera are known to produce several bioactive antibiotics, such as bacillomycin, bacilysin, fengycin, colisin, circulin, polymixin and zwittermicin (Jack et al. 1995; Athukorala et al. 2009; Chen et al. 2009). Although no extensive search for antibiotics has been carried out in these isolates, preliminary experiments have shown that *Bacillus* sp. BMH harbors the *bam*C gene for bacillomycin biosynthesis and *Paenibacillus* sp. 512 carries genes for zwittermicin A resistance (Silva 2012). Isolate 127 was the only Gram-negative bacteria tested and although it carries the pyrrolnitrin gene cluster (Silva 2012), its compatibility with all the other isolates tested indicates either that the antibiotic is not being synthesized at least in vitro or the other bacteria are not sensitive to it. In fact, most strains of *Bacillus* tested to date are not particularly as sensitive to pyrrolnitrin as species of *Streptomyces* or certain fungal species such as *Botrytis cinerea* Pers.:Fr and *Alternaria* sp. (Chernin et al. 1996; El-Banna and Winkelmann 1998).

The data obtained in this study do not allow for any conclusion about the mechanisms of action employed by the bacterial agents to reduce the disease. Nevertheless, it is tempting to speculate that the isolates may have, in some cases, directed their activity, such as competition for nutrients and space, and antibiosis against each other, resulting in limited efficacy of the

Table 3 Efficacy in biocontrol as measured by the reduction in disease incidence due to the application of individual or combined biocontrol agents and the expected values for combined applications of bacterial agents

Treatments	Experiment I		Experiment II	
	Efficacy of biocontrol (%)*	Expected value[a]	Efficacy of biocontrol (%)*	Expected value[a]
512	56.6 ± 11.7 a		44.4 ± 4.44 a	
105	68.3 ± 6.67 a		42.2 ± 2.22 a	
127	20.0 ± 8.66 c		46.6 ± 3.85 a	
90	65.0 ± 8.66 a		46.6 ± 3.85 a	
BMH	36.6 ± 13.3 b		44.4 ± 2.22 a	
INV	68.3 ± 8.82 a		48.8 ± 5.88 a	
512 + 105	33.3 ± 6.01 b	86.3	8.80 ± 5.88 c	67.9
512 + 127	35.0 ± 10.4 b	65.3	11.1 ± 8.01 c	70.4
90 + 105	31.6 ± 1.67 b	88.9	6.60 ± 6.67 c	69.2
127 + 90	35.0 ± 10.0 b	72.0	24.4 ± 2.22 b	71.6
127 + 105	5.00 ± 0.00 c	74.7	51.1 ± 4.44 a	69.2
127 + BMH	25.0 ± 7.64 b	49.3	31.1 ± 9.69 b	70.4
127 + INV	75.0 ± 5.00 a	74.7	48.8 ± 2.22 a	72.7
All isolates	38.3 ± 4.41 b	–	26.6 ± 10.2 b	–
Water	–	–	–	–
Aspergillus	–	–	–	–

According to Limpel's criterion biocontrol efficacies equal or higher than the expected values indicate the existence of synergism between two biocontrol agents

[a]Based upon Limpel's formula for synergism in combinations of two biocontrol agents

*Means followed by the same letter in the columns are not significantly different according to Scott–Knott's tests at 5% probability. Comparisons should be done inside each independent experiment only. Values are means of three replicates of 20 plants per replicate followed by SE

combined applications in the field. Several authors reported lack of synergism in combinations of biocontrol agents (Meyer and Roberts 2002; Xu et al. 2011). In fact, synergistic effects are rare in combinations of biocontrol agents. Only in 2% out of 465 published treatments there was synergy between biocontrol agents according to an investigation carried out by Xu et al. (2011). These authors also affirm that most studies claim the existence of synergism where there is none because no statistical analysis is done to prove it numerically. In this study we used Limpel's criterion that is commonly used to evaluate synergism in combination of chemical pesticides and also biological products (Ippolito et al. 2005; Richter 1987). Theoretical models have indicated that biocontrol agents with different mechanisms of action have a higher chance of acting synergistically than biocontrol agents with the same mechanisms (Xu et al. 2011; Xu and Jeger 2013). It is possible that the strains used in this study possess the same mechanism of action and therefore no synergy resulted in most combinations.

The combined application of the isolates employed in this study is not advantageous because of the lack of synergistic effect and the high cost of registering and formulating two biocontrol agents in one biological product. The registration of each biological control agent needs to be done independently and therefore combinations are required to perform better than individual agents to justify the extra cost (Harman 2000; Guetsky et al. 2001). Nevertheless, single application of any isolate with exception of 127 and BMH, is still a promising strategy to reduce sisal bole rot disease. Isolates 512, 105, 90, INV and the combination between 127 + INV were the most efficacious in decreasing disease incidence, with reductions varying from 42 to 75% and reductions in AUDPC varying from 40 to 94% across two

independent field experiments (Fig. 3). Therefore, on the basis of field experiments, isolates 512, 105, 90, INV may be recommended in single applications against *A. niger*.

The differences in disease incidence and AUDPC levels may be attributed to the complex interactions among microorganisms in soil and the varying temperatures and humidities during the field experiments. Black aspergilli are known to cause other plant diseases such as damping-off of bean and maize, root rot of onion and peanut and seed and seedling rot of *Welwitischiae mirabilis* Hook (Hong et al. 2013; Ismail 2017; Palencia et al. 2010). In all these cases the black aspergilli involved are favored by high temperatures, which are known to cause physiological stresses to plants. Although not many studies have been done in this area, early reports on the pathogenicity of *Aspergillus* indicate that the fungus only infects sisal plants with mechanical and/or physiological injuries (Wallace and Dieckmahns 1952). Once the plant is infected by the pathogen, it will certainly lead to its death. For this reason, evaluations of disease incidence are more appropriate and will reflect the field situation more accurately than disease severity (Zadoks and Schein 1979).

The isolates reported in this study were applied to the plants without any formulation. Formulations suitable to protect the biocontrol agents against the harsh environmental conditions of the Caatinga biome, where sisal is grown, can supposedly improve their field performance by decreasing the variations observed in the field experiments. In this context, Gram-positive bacteria are more amenable for formulation because their endospores allow for longer shelf lives and this should be considered when choosing for isolates. Additionally, the formulations may help the isolates colonize the root surface as well as the plant interior. Biological products formulated with these isolates may contribute to the sustainability of sisal in the Caatinga biome and other regions of the world where this crop is grown.

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