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REGULAR ARTICLE

Rhizobium strains in the biological control of the phytopathogenic fungi *Sclerotium* (*Athelia*) *rolfsii* on the common bean

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Abstract
Aims To identify *Rhizobium* strains' ability to biocontrol *Sclerotium rolfsii*, a fungus that causes serious damage to the common bean and other important crops, 78 previously isolated rhizobia from common bean were assessed.
Methods Dual cultures, volatiles, indole-acetic acid (IAA), siderophore production and *16S rRNA* sequencing were employed to select strains for pot and field experiments.
Results Thirty-three antagonistic strains were detected in dual cultures, 16 of which were able to inhibit ≥84% fungus mycelial growth. Antagonistic strains produced up to 36.5 μg mL^{-1} of IAA, and a direct correlation was verified between IAA production and mycelium inhibition. *SEMIA* 460 inhibited 45% of mycelial growth through volatile compounds. *16S rRNA* sequences confirmed strains as *Rhizobium* species. In pot condition, common bean plants grown on *S. rolfsii*-infested soil and inoculated with *SEMIA* 4032, 4077, 4088, 4080, 4085, or 439 presented less or no disease symptoms. The most efficient strains under field conditions, *SEMIA* 439 and 4088, decreased disease incidence by 18.3 and 14.5% of the *S. rolfsii*-infested control.
Conclusions *Rhizobium* strains could be strong antagonists towards *S. rolfsii* growth. *SEMIA* 4032, 4077, 4088, 4080, 4085, and 439 are effective in the biological control of the collar rot of the common bean.

Keywords Crop improvement · Indole-acetic-acid · Pathogenic fungus · *Phaseolus vulgaris* · Rhizobia

Abbreviations

IAA	Indole-acetic-acid
MAPA	Ministry of Agriculture, Livestock, and Supply
AUDPC	Area under the disease progress curve

Introduction

The common bean (*Phaseolus vulgaris* L.) is considered one of the most important grain legumes produced worldwide. Brazil, China, India, Mexico, Myanmar, and the USA are the most important *Phaseolus* spp.-producing countries (FAO 2014). As for other crops,

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common bean cultivars have high nutritional requirements and require proper phytosanitary conditions to express their full genetic potential. In this way, microbial inoculation technologies have been studied to decrease the environmental impacts and production costs of agrochemical inputs on crops.

Rhizobia are bacteria that can induce root nodules on leguminous plants and convert atmospheric nitrogen into available ammonia (Lindström and Martinez-Romero 2005), and therefore are a widely used microbial inoculant. Pelegrin et al. (2009) and Soares et al. (2006) evaluated the *Rhizobium tropici* strain *SEMIA* 4077 and obtained common bean yields equivalent to the application of 70–80 kg ha^{-1} of nitrogen. Rhizobia can also stimulate both leguminous and non-leguminous growth via: the secretion of molecules analogous to plant hormones, such as auxins (i.e., IAA; Ghosh et al. 2015); solubilization of inorganic insoluble phosphates (Kumar and Ram 2014); mineralization of organic phosphates (López-López et al. 2010); improving iron acquisition and/or alleviating aluminum toxicity mediated by chelating molecules known as siderophores (Datta and Chakrabartty 2014; Roy and Chakrabartty 2000); decreasing ethylene inhibitory action via ACC deaminase activity (Glick 2005); and the induction of abiotic stress tolerance by secreting exopolysaccharides and formatting biofilm structures (Qurashi and Sabri 2012).

The legislation about the use of inoculants as biofertilizers are currently under development in the European Union and the USA (Malusá and Vassilev 2014). Since 1975, in Brazil, the Ministry of Agriculture, Livestock, and Supply (*Ministério da Agricultura, Pecuária e Abastecimento*, abbreviated as MAPA) regulated the commercialization of rhizobial inoculants. *SEMIA*, an official culture collection, was assigned to maintain the recommended bacterial inoculant strains for important crop species and also distribute those strains to the inoculant industry. Now, *SEMIA* 4077 (= CIAT 899 = UMR 1899 = HAMBI 1163 = DSM 11418 = ATCC 49672 = LMG 9503 = USDA 9030), *SEMIA* 4080 (= PRF 81), and *SEMIA* 4088 (= H 12) are the recommended strains for common bean inoculation in Brazil. *SEMIA* also contains hundreds of strains for which no recommendation status was designed due to their low efficiency in nitrogen fixation.

Besides their nitrogen-fixing ability, rhizobia could also be used as biological control agents, especially against phytopathogenic fungi, such as *Fusarium solani* (Dar et al. 1997), *F. oxysporum*, *Rhizoctonia solani* (de Jensen et al. 2002), *Phytium* sp. (Huang and Erickson 2007), and *Verticillium* sp. (Vargas et al. 2009). Biocontrol agents act through different mechanisms, such as: antibiotics (Robleto et al. 1998) and HCN (Chandra et al. 2007) action; nutrient competition (siderophores are often involved); action of extracellular cell wall-degrading enzymes, i.e., β-1,3-glucanases, proteases (Compant et al. 2005), and chitinases (Kacem et al. 2009); parasitization of hyphal tips and inhibition of reproductive structures, like sclerotia or zoospores; and detoxifying a pathogen's virulence molecules, such as oxalic acid (Nagarajkumar et al. 2005) and hydrolytic enzymes. In addition, rhizobia could exert "indirect antagonism" by eliciting plant defense reactions (Elbadry et al. 2006).

Sclerotium rolfsii (teleomorph: *Athelia rolfsii*) is a necrotrophic, ubiquitous soil-borne phytopathogenic fungus responsible for severe crop losses, particularly in tropical and subtropical regions. Regarding the common bean, this fungus is known to promote collar rot disease, leading to complete wilting of the affected plants (Bianchini et al. 1997). Controlling *S. rolfsii* using chemicals is difficult for many reasons, such as dissemination of the fungus by contaminated equipment and machinery, the fungus' ability to form sclerotia (Punja and Grogan 1981), and the extensive fungal host range of at least 500 plant species (Iquebal et al. 2017). Also, fungicide utilization leads to the killing of non-target beneficial microorganisms and contributes to water and soil pollution.

The extensive information available on rhizobial application and the potential among the vast number of strains/isolates stored in different culture collection centers for use as biocontrols could lead to rhizobia at least partially replacing agricultural pesticides. Therefore, the aim of this study was to reevaluate the *SEMIA Culture Collection* with a focus on identifying the first effective *Rhizobium* biocontrol agent for the collar rot disease of the common bean.

Materials and methods

Organisms, culturing media, and conditions

Bacteria previously isolated from common bean nodules and maintained at *SEMIA Culture Collection* (World Data Center on Microorganisms no. 443) were used in this study. Lyophilized bacteria were rehydrated and

grown on YMA medium plates (Somasegaran and Hoben 2012) at 28 °C. Whenever a log-phase liquid culture was required, bacteria were multiplied in 15-mL tubes containing 5 mL of YM broth, which were placed in a rotary shaker (120 rpm) for 72 h.

The fungal pathogen was previously isolated from common bean plants cultivated at Viamão Research Center (geographic coordinates: 30°2′10.64”S and 51°1′ 17.65”W), at the Agriculture Department of Rio Grande do Sul State, located in Viamão, Brazil. The pathogen was identified as *S. rolfsii* based on its etiology and morphological characteristics (Mordue and Holliday 1974). *S. rolfsii* cultures were obtained by cultivation on PDA medium (Beever and Bollard 1970) for five days at 23 °C and with a 12-h photoperiod. Fungal identity was further confirmed by PCR amplification of an internally-transcribed spacer region using the ITS1/ITS4 universal primer (Kawasaki 1990). The amplified PCR product was sequenced and a BLASTN 2.7.0+ (Altschul et al. 1997) search using a query length of 597 revealed 99% homology to *Sclerotium* (*Athelia*) *rolfsii* (GenBank MF425542.1).

Dual culture assay

The screening of 78 bacterial strains from the *SEMIA Culture Collection* for *S. rolfsii* antagonism was performed based on a dual culture method on TY medium plates (Somasegaran and Hoben 2012). A 0.7-cm agar plug from a *S. rolfsii* culture was placed at the center of a 9-cm plate and the bacterium was streaked in a square form and incubated for five days at 23 °C and with a 12-h photoperiod (Supplementary Figure 1). Inhibition is described in percentages according to the equation (%) = [(C − 0.7) − (T − 0.7) / (C − 0.7)] × 100, where C is the mean of the fungal colony diameter (cm) from solely cultivated fungal plates and T is the fungal colony diameter (cm) in the dual culture. Each *SEMIA* strain was tested on three different plates.

Indole-acetic acid, siderophore, protease and cellulase detection

Antagonistic *SEMIA* strains detected in the dual cultures were tested in triplicate for IAA, siderophore, protease, and cellulase production. IAA production was evaluated based on the method of Asghar et al. (2002). Bacterial log-phase liquid cultures in 0.5 g L^{-1} tryptophan-supplemented broth were centrifuged for 5 min at 10,000 rpm and 500 μL of the supernatants were placed in microtubes to react with 500 μL of Salkowski reagent (2 mL 0.5 mol L^{-1} $FeCl_3$ + 98 mL 35% $HClO_4$). The mixture was left in the dark for 15 min at room temperature, and subsequent spectrophotometer measurements were taken at 520 nm. The IAA concentration was inferred from a standard curve.

The effect of exogenous IAA on *S. rolfsii* growth was also measured. Different IAA concentrations (0, 0.5, 5, 50, 500, or 5000 μM) were added to TY medium and a 0.7-cm agar plug from a *S. rolfsii* culture was placed at the center of a 9-cm plate. TY medium without IAA served as a control. Plates were incubated for five days at 23 °C and with a 12-h photoperiod. IAA inhibition is described in percentages according to the equation (%) = [(C − 0.7) − (T − 0.7) / (C − 0.7)] × 100, where C is the fungal colony diameter (cm) in the TY medium and T is the fungal colony diameter (cm) in the IAA-supplemented TY medium. Each IAA concentration was tested on five different plates.

The proteolytic and cellulolytic activities of antagonistic strains were inferred by the detection of hydrolyzed zones on agar plates containing skim milk (Montanhini et al. 2013) or carboxymethyl cellulose (Kasana et al. 2008) after inoculation with 5-μL drops of bacterial log-phase liquid cultures.

For siderophore detection, the bacterium strain was multiplied in 100% and 1:2 water diluted with iron-deficient liquid King’s B medium as previously described (Schwyn and Neilands 1987). Subsequently, cultures were centrifuged for 5 min at 10,000 rpm and an aliquot of 50 μL was collected and dropped onto a microplate along with 50 μL of chrome azurol-S (CAS) reagent, followed by incubation for 15 min. Bacterial strains that changed the color of the reaction mixture from blue to orange were considered positive for siderophore production. Antagonistic activity related to siderophores was determined through the comparison of iron-deficient King’s B-treated cultures with ones supplemented with 100 μM of $FeCl_3$ (Bevivino et al. 1998) in dual culture assays.

Volatile detection through the double plate assay

Antagonistic *SEMIA* strains with different characteristics were selected for further testing. Production of volatile compounds that inhibited fungal mycelial growth was detected via the double plate assay. A 0.7-cm agar plug from a *S. rolfsii* culture was placed at the center of a PDA

medium plate. Subsequently, 30 μL of bacterial log-phase liquid culture were spread on Congo Red YM medium plates. Petri dishes containing the fungal plug were then placed inverted over the plate with the bacterium, and control treatments were prepared without the bacteria. The plates were incubated for five days at 23 °C and with a 12-h photoperiod. Each *SEMIA* strain was tested on three different plates. Inhibition through volatiles is presented in percentages according to the inhibition equation used for dual cultures.

16S rRNA sequence analysis

The genomic DNA of each selected *SEMIA* strain was extracted and purified according to the method of Joseph and David (2001). The *16S rRNA* was amplified using the BacPaeF (5'AGA GTT TGA TCC TGG CTC AG3') and Bac1542R (5'AGA AAG GAG GTG ATC CAG CC3') primers, according to the methods of Stackebrandt and Liesack (1993) and Edwards et al. (1989), in a final volume of 25 μL containing 20–50 ng of genomic DNA, 1 μL of 100 pmol of each primer, 1 μL of 0.25 mmol L^{-1} dNTP mix, 1 μL of 50 mmol L^{-1} $MgCl_2$, 1 μL of DMSO, 2.5 μL of Taq DNA Polymerase PCR Buffer (10×), and 0.2 μL of Taq DNA Polymerase (Thermo Scientific). The PCR cycling program was: 94 °C for 5 min, followed by 37 cycles of 94 °C for 1 min, 50 °C for 1 min and 10 s, and 72 °C for 1 min; for the final step, the reaction was incubated at 72 °C for 5 min. Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, South Korea) using an ABI3730XL. Low-quality sequences were trimmed using Chromas 2.6.4 software.

Sequence identity was assessed by comparing the *16S rRNA* sequences of the *SEMIA* strains with the sequences from EzBioCloud (https://www.ezbiocloud.net/identify), a quality-controlled *16S rRNA* server database.

The *16S rRNA*-based phylogeny was construct employing *16S rRNA* sequences from *SEMIA* and 111 *Rhizobium* type strains available in LPSN (available at http://www.bacterio.net). Sequences were aligned with MUSCLE on MEGA7 software. Bayesian analyses was prepared using BEAST v1.8.4 software. HKI was selected as the mode of nucleotide evolution. The Yule process was selected as a tree prior to Bayesian analysis. The MCMC algorithm ran for 10,000,000 generations. The trees were visualized and edited using FigTree 1.4.3 software.

The sequence data reported in this study are publicly deposited in GenBank under accession no. MH236581-MH236590.

Biocontrol pot and field experiments

S. rolfsii inoculum was made according to the method of Falcão et al. (2005), with slight modifications. Erlenmeyer flasks containing moistened (20% m/v) maize (*Zea mays* L.) grains were autoclaved at 120 °C for 20 min. Afterward, 0.7-cm agar plugs from a *S. rolfsii* culture were placed in the flask and then incubated for 14 days at 23 °C and with a 12-h photoperiod. Pre-sprouting common bean cv. FEPAGRO Triunfo (Brazilian National Register of Cultivars no. 31.376) seeds were obtained after placing the seeds in moistened filter paper following incubation in germination chambers for 2 days (16 h of light at 30 °C and 8 h of dark at 20 °C). Pre-sprouting seeds were immersed in bacterial log-phase liquid cultures for 5 min. Subsequently, 180-mL pots were filled with non-autoclaved field soil, with the following characteristics: pH = 5.40, organic matter = 0.4%, CEC pH 7 = 3.5 and effective CEC (ECEC) = 1.8, p = 42.9 mg/dm^{-3}, K = 41 mg/dm^{-3}, Al = 0.1 $cmol_c/dm^{-3}$, Ca = 1.1 $cmol_c/dm^{-3}$, Mg = 0.5 $cmol_c/dm^{-3}$, H + Al = 1.7 $cmol_c/dm^{-3}$, B = 0.4 mg/dm^{-3}, Zn = 3.2 mg/dm^{-3}, Cu = 0.3 mg/dm^{-3}, Mn = 29 mg/dm^{-3}, Na = 4 mg/dm^{-3}, Arg = 7%, and Fe = 0.04%. Soil infestation was made with two *S. rolfsii*-infected maize grains that were placed along the pots, at the same depth and on each side of one bacteria-inoculated pre-sprouting common bean seed (Supplementary Figure 2). Two control treatments were arranged. Treatment "-" (negative control) was composed of common bean plants that were not inoculated with bacteria, which were allowed to grow in non-infested soil. "Treatment +" (positive control) was composed of common bean plants that were not inoculated with bacteria, but were cultivated in *S. rolfsii*-infested soil. Three replicates were used per treatment. The plants were inspected periodically to determine disease manifestation development. Shoot dry masses were recorded through separation of the aerial part of each plant after drying at 60 °C to a constant mass.

Rhizobial strains were selected for field trial based on pot test results. Field experiment was carried out at Viamão Research Center. For this, plastic bags containing 30 common bean seeds were inoculated with 2 mL of bacterial log-phase liquid cultures before being sown on a soil. The

experiment was conducted in a randomized block design with 4 replications. Plot size was 1.2 m × 2 m with 40 cm line-to-line spacing. The space between the blocks and plots were both 1 m each. Despite the soil being already naturally infested with *S. rolfsii*, to guarantee disease manifestation the common bean seeds were sown intercalated with *S. rolfsii*-infected maize grains as described before, and then covered with soil with the following characteristics pH = 6.0, organic matter = 2.2%, CEC pH 7 = 9.4 and effective CEC (ECEC) = 5.0, $p = 5.4$ mg/dm^{-3}, K = 123 mg/dm^{-3}, Al = 0.0 cmol$_c$/dm^{-3}, Ca = 3.3 cmol$_c$/dm^{-3}, Mg = 1.4 cmol$_c$/dm^{-3}, H + Al = 4.4 cmol$_c$/dm^{-3}, B = 0.2 mg/dm^{-3}, Zn = 4.9 mg/dm^{-3}, Cu = 0.8 mg/dm^{-3}, Mn = 12.5 mg/dm^{-3}, Na = 3 mg/dm^{-3}, Arg = 24%, and Fe = 0.3%. A bacterial-uninoculated control treatment ("Treatment +") was also arranged. No plant protection measure for controlling diseases of the crop or nitrogen fertilizer was applied. The experimental plots were inspected periodically to determine disease manifestation development. At the beginning of the flowering stage (around 40 days after being sown) disease incidence was recorded according to the equation disease incidence (%) = (number of completely wilted plants / number of germinated plants) × 100. A disease progress curve was plotted and the area under the disease progress curve (AUDPC) for each treatment was estimated.

Statistical analysis

Using the Sisvar 5.6 platform (Ferreira 2011), the results obtained from the dual culture, double plate, exogenous IAA influence on fungal growth, and biocontrol tests were submitted to one-way analysis of variance and the means were compared by the Scott-Knott (SK) test at a 5% error probability. Spearman's correlation was used to evaluate IAA production and fungal mycelium inhibition on dual cultures. Data from evaluating the antagonistic activity related to siderophores were submitted to the F test, and then the means were compared by Student's t test at the 5% level of significance.

Results

SEMIA rizobial strains strongly antagonize *S. rolfsii* growth

Dual cultures were performed to screen the *SEMIA Culture Collection* for biocontrol properties against the pathogenic fungus *S. rolfsii* (Fig. 1a). Among the 78 screened strains, 33 (~42%) of them showed significant antagonistic activity, and 16 were able to decrease over 84% of the fungal mycelium growth. *SEMIA* 456, 4026, and 436 presented inhibition rates above 98% of mycelia growth. The MAPA-recommended strains *SEMIA* 4080, 4077 and 4088 were able to decrease the mycelial growth by 86%, 86%, and 60%, respectively.

Bacterial IAA production correlates with *S. rolfsii* inhibition

In vitro, the antagonistic *SEMIA* strains produced 1.2 to 36.5 μg mL^{-1} of IAA. The MAPA-recommended strains *SEMIA* 4077, 4080, and 4088 produced 2.5, 6.1, and 4.7 μg mL^{-1} of IAA, respectively. The prominent IAA producers, *SEMIA* 456 (34.7 μg mL^{-1}) and 439 (36.5 μg mL^{-1}), were grouped along with major *S. rolfsii* antagonists in dual cultures. A significant correlation ($r = 0.447$, $p = 0.011$) was obtained between bacterial IAA production and *S. rolfsii* mycelial growth inhibition in the dual cultures (Fig. 1b). The effect of exogenous IAA on the growth of *S. rolfsii* was studied (Fig. 1c). TY medium supplemented with 50 μM (8.8 μg mL^{-1}) of IAA had no effect on fungal growth; however, *S. rolfsii* growth was decreased at IAA concentrations of 250 (43.8 μg mL^{-1}) and 500 μM (87.6 μg mL^{-1}). The highest concentration (5000 μM or 876 μg mL^{-1}) of IAA inhibited over 99% of fungal growth.

Some *SEMIA* strains produce lytic enzymes and siderophores

Among the 33 antagonistic strains found, siderophore production was detected only in *SEMIA* 436, 460, 4077, and 4088. A 100-μM $FeCl_3$ supplement upon growth is expected to inhibit siderophore compound synthesis (Visca et al. 1992). In order to evaluate if the *SEMIA* strains' antagonistic activity on *S. rolfsii* mycelium growth was related to the iron availability in the growth media, dual cultures with either iron-deficient or $FeCl_3$-supplemented King's B medium were performed (Fig. 2a). Dual cultures with *SEMIA* 4088 plates in $FeCl_3$-supplemented medium had 13% greater mycelium spread than iron-deficient plates; no other statistically significant differences in mycelium growth were found. Lytic enzyme presence was also tested, and *SEMIA* 4026, 4031, and 4079 were the only strains that produced proteases; cellulase production was not detected (*data not shown*).

a)

SEMIA strain	*S. rolfsii* mycelium inhibition %	IAA production µg mL^{-1}
456	99	34.7
4026	99	3.3
436	98	18.0
439	95	36.5
457	94	20.6
4032	93	22.3
464	93	16.6
461	92	15.5
4033	92	10.9
462	90	4.6
4031	89	16.8
480	87	13.5
4080	86	6.1
472	86	12.8
4077	86	2.5
465	84	3.6
4079	81	1.2
4022	78	5.0
4084	78	1.8
455	76	17.3
4016	76	4.1
4036	76	5.0
4070	73	2.0
463	70	6.2
481	65	7.3
4039	65	1.2
4088	60	4.7
485	58	4.8
460	51	9.2
4029	50	21.7
449	49	7.5
4085	10	1.8
422	5	10.7

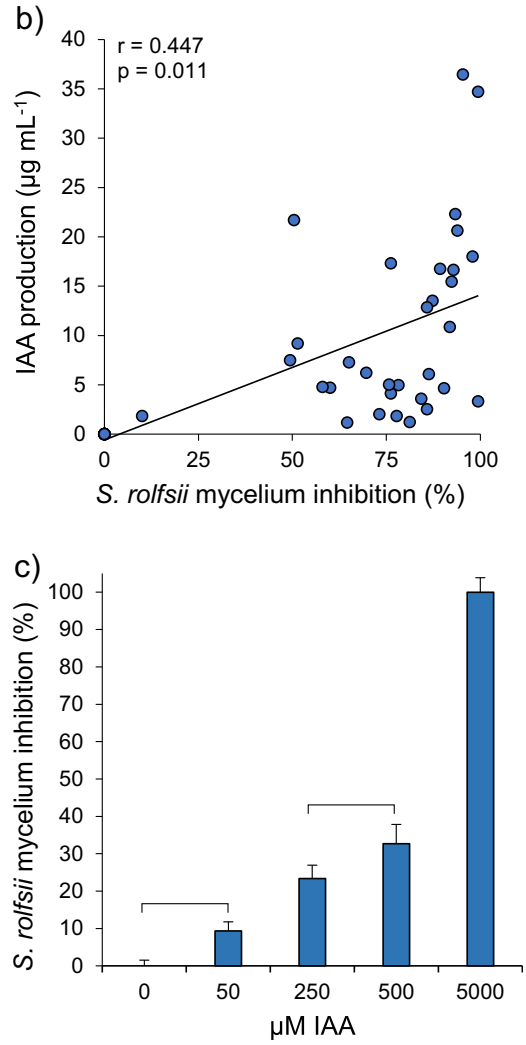

Fig. 1 **a** Effect on *S. rolfsii* mycelial growth and indole-acetic acid (IAA) production of bacterial strains from *SEMIA* culture collection. Seventy eight bacterial strains stored in the *SEMIA Rhizobium Culture Collection* were screened for their ability to antagonize *S. rolfsii* growth in dual cultures. The antagonistic strains were also tested for IAA production in liquid cultures. Data in brackets do not differ by the Scott-Knott (SK) test at 5% error probability. **b** Scatter plot between bacterial IAA production and ability to inhibit *S. rolfsii* mycelium growth. Spearman's $r = 0.447$, p value = 0.011. **c** Effect of exogenous IAA on *S. rolfsii* growth. Fungus was grown on TY medium supplemented with different IAA concentrations. Error bars represent the standard error of the mean. Data in brackets do not differ by the SK test at 5% error probability

For further testing, we selected the antagonistic strains i) *SEMIA* 456 (= F 33 = D19 RIC*p* = Br 209), *SEMIA* 4026 (= CAR 29), *SEMIA* 436 (= F 27), *SEMIA* 439 (= F 35 = D 71) and *SEMIA* 4032 (= TAL 659 = ALLEN 413–2 = UW 4032) due to their strong fungal inhibition (≥93%) and IAA production features; ii) *SEMIA* 460 (= F 37) was selected as a median inhibitor (51%) and for the presence of a mucoid colony phenotype (often an indication of enhanced biofilm formation with possible biocontrol implications, according to Chen et al. (2013); iii) *SEMIA* 4085 (= USDA 2918 = R602s*p* = EMBRAPA Soja 172) was selected as a poor inhibitor (10%); and iv) *SEMIA* 4080, 4077 and 4088 because they are MAPA-recommended strains.

Some *SEMIA* strains antagonize *S. rolfsii* growth through the production of volatile compounds

Selected antagonistic *SEMIA* strains were tested, employing the double plate technique, for the production

of volatile substances that could inhibit fungal growth (Fig. 2b). *SEMIA* 460, 4077, and 4088 decreased mycelium diameters by 45%, 28%, and 28% through the production of volatile compounds, respectively.

16S rRNA sequence similarity analysis and phylogenetics of *SEMIA* strains

The genus of the *SEMIA* strains was discovered/confirmed by similarity-based searches against quality-controlled databases of *16S rRNA* sequences with EzBioCloud's Identify service (Supplementary Table 1). All *SEMIA* strains presented higher gene similarities with species belonging to the *Rhizobium* genus.

A phylogenetic tree was generated with *SEMIA* sequences and the *Rhizobium*-type strains (Fig. 3). *SEMIA* 4077 (*R. tropici*), 4080, and 4088 (*R. freire*) were grouped along with *R. hainanense*, *R. miluonense*, and *R. multihospitium*; *SEMIA* 4085 (*R. gallicum*) and *SEMIA* 460 were grouped with *R. anhuiense, R. trifolii, R. leguminosarum, R. acidisoli,* and *R. laguerreae*; and *SEMIA* 436, 439, 456, 4026, 4032 were grouped with *R. radiobacter* and *R. pusense*.

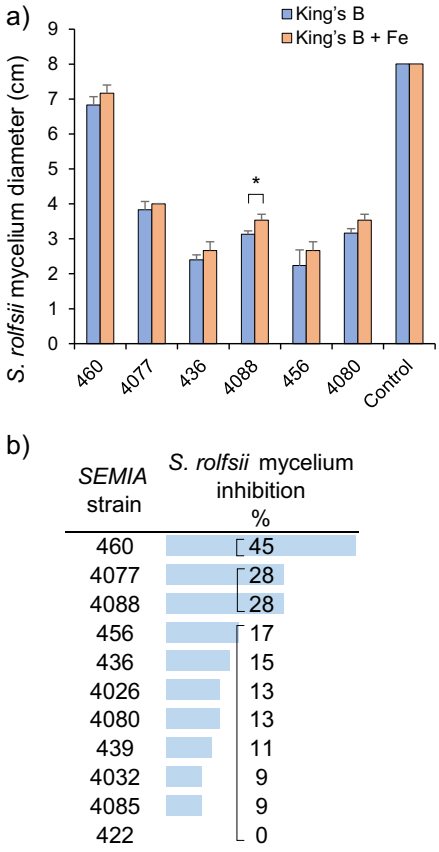

Fig. 2 **a** Siderophore influence on *S. rolfsii* mycelium growth from antagonistic *SEMIA* bacterial strains. Dual cultures of both iron-deficient and $FeCl_3$-supplemented King's B medium were performed to identify antagonistic bacterial siderophores' influence on fungal mycelium growth. Data with an asterisk were statistically significantly different (Student's t test at 5%) between cultures grown in iron-deficient and those in $FeCl_3$-supplemented King's B medium. Error bars represent the standard error of the mean. **b** Antagonistic activity of volatile compounds produced by antagonistic *SEMIA* bacterial strains on *S. rolfsii* mycelia. Selected *SEMIA* strains were tested with the double plate technique for the production of antagonistic volatile substances that inhibited *S. rolfsii* growth. Data in brackets do not differ by the SK test at 5% error probability

SEMIA strains exhibit biocontrol efficacy on collar rot of the common bean

In order to provide preliminary data on *SEMIA* strains activities *in planta*, a biocontrol pot experiment was first conducted to select strains for a field test. The common bean cultivated in *S. rolfsii*-infested soil and inoculated with *SEMIA* 4032, 4077, 4088, 4080, or 4085 strains presented no symptoms of collar rot (Fig. 4a). Moreover, treatments with *SEMIA* 4032, 4077, 4088, 4080, 4085, or 439 presented similar shoot dry masses of the uninoculated plants grown on uninfested soil according to SK test at a 5% error probability (Fig. 4b). The common bean inoculated with *SEMIA* 4026, 436, 460, or 456 strains exhibited stem rot symptoms and present similar shoot dry masses of the uninoculated plants grown on infested soil.

In the field trial, the disease incidence was attenuated with *SEMIA* 4032, 4077, 4088, 4080, 4085, or 439 strains treatments (Fig. 5a). The most efficient strains detected, *SEMIA* 439 and *SEMIA* 4088, decreased 18.3 and 14.5% of the *S. rolfsii*-promoted disease incidence, respectively. The remnants strains were able to decrease disease incidence by 12.5 to 8.7%. Since disease incidence was recorded over the time, the AUDPC (Fig. 5b) was estimated. Only the *SEMIA* 439 and *SEMIA* 4088 treatments presented AUDPC values statistically significantly lower from the uninoculated treatment. *SEMIA* 439 and 4088 decreased 19.9 and 17.5% of the AUDPC comparing to uninoculated controls, respectively.

Discussion

In an attempt to promptly obtain a biocontrol agent against *S. rolfsii*-induced disease in the common bean,

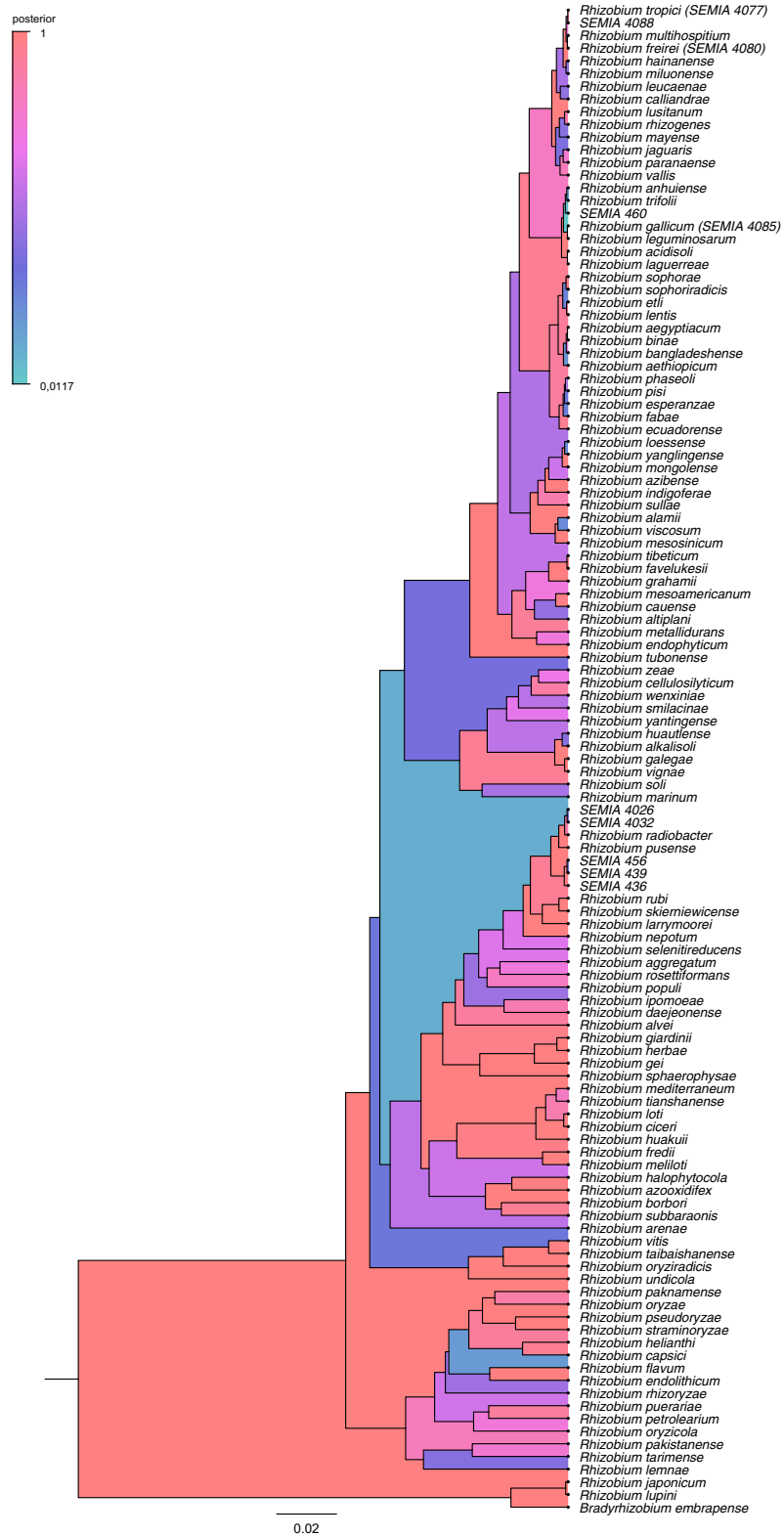

Fig. 3 Phylogenetic tree of *16S rRNA* gene sequences from selected antagonistic *SEMIA* strains and 111 *Rhizobium*-type strains, as inferred by Bayesian analysis. The significance of each branch is indicated at the branching points by posterior probability. HKI was chosen as the mode of nucleotide evolution. The Yule process was selected as a tree prior to Bayesian analysis. The MCMC algorithm ran for 10,000,000 generations

we evaluated bacterial strains from *SEMIA*, a previously stabilized culture collection. Dual culture screens have been used as an effective approach to prospect biocontrol agents and some authors reported bacterial and fungal antagonism toward *S. rolfsii* mycelium growth in dual-culture experiments. Rhizobial isolates from groundnut (*Arachis hypogaea* L.) inhibited up to 62.5% of the *S. rolfsii* mycelium growth diameter (Ganesan et al. 2007). Shaban and El-Bramawy (2011) reported 85.5% mycelial inhibition of *S. rolfsii* when they evaluated a *Rhizobium leguminosarum* isolate. *Bacillus tequilensis* (Gholami et al. 2014), *Trichoderma harzianum* (Sabet et al. 1998), and *Trichoderma viride* (Manjula et al. 2004) were able to inhibit 73.6%, 45%, and 58% of *S. rolfsii* mycelium growth, respectively. In the present work, the prominently antagonistic *SEMIA* strains demonstrated up to 99% inhibition of *S. rolfsii* mycelium growth in dual cultures.

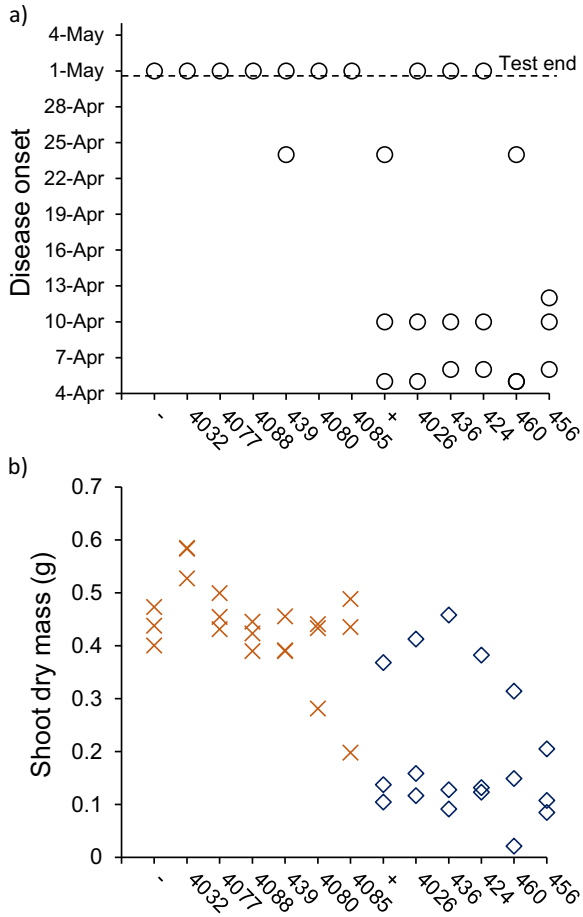

Fig. 4 Biocontrol of *S. rolfsii*-induced disease of the common bean cultivated in pots by employing inoculation with selected *SEMIA* antagonistic strains. **a** Date of onset of the wilt caused by *S. rolfsi.* Each data point represent a common bean plant tested. Data points above the dotted line were not detected with disease symptoms. Treatment "-" (negative control) was composed of common bean plants that were not inoculated with bacteria and grew in uninfected soil. "Treatment +" (positive control) was composed of common bean plants that were not inoculated with bacteria but were cultivated in *S. rolfsii*-infected soil. **b** Shoot dry masses. Data points with a different shape or color differ by the SK test at 5% error probability

Antagonistic *SEMIA* strains produced IAA concentrations similar to that of a previously reported *Rhizobium* spp. that showed beneficial effects on plants (Bhattacharjee et al. 2012). IAA synthesis is considered a common feature in soil-beneficial bacteria and part of their plant colonization strategy. IAA is often considered one of the most effective plant-growth inducers (Vargas et al. 2017). IAA produced by rhizobia is involved in the nodulation process (Boiero et al. 2007; Pii et al. 2007) and root architecture modification (Yanni et al. 2001); however, high IAA concentrations have shown an unresponsive effect and/or adverse impact on plant growth. As an example, Schlindwein et al. (2008) reported lettuce seeds with abnormal germination when those seeds were treated with an IAA-overproducing (171.1 $\mu g.ml^{-1}$) *R. trifolii* strain.

In a biocontrol context, the phytostimulation action of IAA produced by beneficial bacteria could be helpful; however, this action relies on the plant. Exogenous IAA exerts stimulatory and inhibitory effects on fungi (Fu et al. 2015). IAA was also reported to trigger protection against external adverse conditions by enhancing different cellular defense systems in *Escherichia coli* (Bianco et al. 2006). Here, we hypothesized a direct relationship between in vitro bacterial IAA production and *S. rolfsii* mycelial growth inhibition and found a significant ($p = 0.011$) but weak correlation ($r = 0.447$). Kulkarni et al. (2013) reported that 0.5, 5, and 50-μM concentrations of exogenous IAA can induce *Fusarium delphinoides* growth. However, at higher concentrations (500 and 5000 μM), IAA considerably decreased the growth of this fungus. Here, we found that 250 and 500 μM of IAA supplementation to the medium decreased fungal growth, while 5000 μM almost ceased it. Interestingly, *SEMIA* 439, the most preeminent IAA producer detected here, also grouped along the greater fungal inhibitors, produced 208 μM IAA. This result points out a function in soil competitiveness for beneficial bacterial IAA, not merely the improvement of plant-bacteria interaction fitness. It

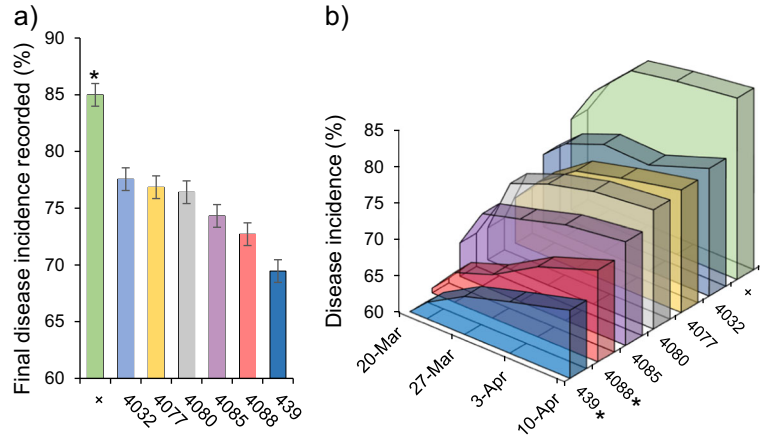

Fig. 5 Biocontrol of *S. rolfsii*-induced disease of the common bean cultivated in field by employing inoculation with selected *SEMIA* antagonistic strains. **a** Disease incidence considering the final sampling recording. Data with asterisk differ from other treatments by the SK test at 5% error probability. Error bars represent the standard error of the mean. **b** Area under the disease progress curve (AUDPC). Treatments with the asterisk differ from other treatments by the SK test at 5% error probability. "Treatment +" (positive control) was composed of common bean plants that were not inoculated with bacteria but were also cultivated in *S. rolfsii*-infected soil

remains to be elucidated if IAA has a secondary role stimulating responses in the prokaryotic cell, besides its direct impact on fungal mycelium growth.

Besides IAA action, biological control agents can negatively affect the growth of plant pathogens by several other mechanisms. For example, Rodriguez-Kabana et al. (1978) reported that the proteolytic activity of *Trichoderma viride* was crucial for disrupting *S. rolfsii*'s enzymatic activity. Therefore, we tested protease exportation to the culture medium and found this characteristic in only three (~9%) of the 33 antagonistic *SEMIA* strains. Siderophore-producing antagonistic rhizobia were also reported (Granada et al. 2014; Vargas et al. 2009); however, siderophore production is not always related to antagonistic activity, as was already reported regarding *Burkholderia cepacia* (Bach et al. 2016). Genome sequencing revealed that *SEMIA* 4077 possesses a siderophore-biosynthesis gene cluster, whereas *SEMIA* 4080 does not (Ormeño-Orrillo et al. 2012). In agreement, *SEMIA* 436 and 460 and the MAPA-recommended strains *SEMIA* 4077 and 4088 were identified as siderophore producers. However, *SEMIA* 4088 was the only strain that demonstrated a slightly antagonistic activity related to siderophore production.

In addition to the production of diffusible antifungal molecules, such as lytic enzymes and siderophores (Kümmerli et al. 2014), some bacteria also synthesize volatile compounds that influence fungal growth (Bhagat et al. 2014; Wheatley 2002). As an example, soil bacterial volatiles were reported to completely stop the laccase activity of *Phanerochaete magnolia* (Mackie and Wheatley 1999). Laccase is an important virulence factor for various phytopathogenic fungi because it protects them from plant defense molecules, such as tannins and phytoalexins (Pezet et al. 1992). Ganesan et al. (2007) reported inhibition of up to 11% of *S. rolfsii* growth by volatiles produced by *Rhizobium* species. Here, *SEMIA* 460 was the major volatile inhibitor (45%); these volatiles were likely its major antagonism mechanism, considering the 51% inhibition detected in the dual cultures.

Bacterial strains from the *SEMIA Culture Collection* were previously identified as *Rhizobium* spp. based on prospecting from common bean root nodules and cultural characteristics. To confirm that bacterial identification, *16S rRNA* sequence analysis was employed. To provide reliable identification since it may be compromised by the quality of sequences deposited in public databases (i.e., NCBI), the curated *16S rRNA* database EzBioCloud (Yoon et al. 2017) was chosen for comparisons and the *Rhizobium* spp. identities of the *SEMIA* 456, 4026, 436, 439, 4032, 460, 4085, 4080, 4077, and 4088 strains were confirmed. As expected, although *16S rRNA* analysis allows the identification of the

Rhizobium genus, this high degree of gene conservation seriously limited the separation at the species level.

The *SEMIA* 4077 and 4080 sequences that we obtained presented highly similar (99%) *16S rRNA* gene sequences. Often considered one of the most successful symbionts of the common bean, *SEMIA* 4077 is the type strain of the *Rhizobium tropici* species (Martínez-Romero et al. 1991). *SEMIA* 4080, which in the past was considered a *Rhizobium tropici* strain, shares only 52% of its genome sequence with *SEMIA* 4077 (Ormeño-Orrillo et al. 2012). Recently, *SEMIA* 4080 was recognized as the first *Rhizobium freirei* strain described (Dall'Agnol et al. 2013).

In the phylogenetic tree with 111 *Rhizobium*-type strain sequences, *SEMIA* 436, 439, 456, 4026, and 4032 were placed in a branch with *R. pusense* and *R. radiobacter* (*Agrobacterium tumefaciens*). These bacteria, which could be placed on controversial *Agrobacterium* genus (Ramírez-Bahena et al. 2014; Young et al. 2001), are usually known for being tumorigenic; therefore, they would not be expected to form nitrogen-fixing nodules. However, Ribeiro et al. (2013) reported strains related to *R. radiobacter*, *R. nepotum*, and *R. pusense* according to *16S rRNA* analysis, which were isolated from the common bean and could form white nodules on roots.

Nodulation (*nod*) and nitrogen-fixation genes (*nif* and *fix*) are often clustered on large plasmids (Sym plasmid) or within genomic symbiosis islands (SI), which are often found within insertion sequence elements, transposases, and related genes (MacLean et al. 2007). Root-nodulating bacteria that, according to ribosomal gene analysis, were related to "non-rhizobial" species/genus have been found to naturally harbor the *nod* genes essential for establishing rhizobial symbiosis (Moulin et al. 2001; Trujillo et al. 2006). Martínez et al. (1987) engineered agrobacteria to harbor the Sym plasmid and obtained a mutant that could form nitrogen-fixing nodules. In addition, agrobacteria could lose their tumorigenic characteristics, i.e., high temperature (>28 °C) culturing leads to "loosening" of the Ti plasmid in the bacterial population (Schilperoort et al. 1980).

In this work, dual culture screens were used to prospect an efficient bacterial biocontrol agent of *S. rolfsii*-induced disease of the common bean. Dual cultures are widely used for this purpose and according to Shehata et al. (2016) the results strongly correlate with microbial activities *in planta*. However, *SEMIA* 4026, 436, and 456 were strong in vitro fungal antagonists but did not succeed in controlling the disease *in planta*. A dual culture-screened isolate may not succeed *in planta* for many reasons (i.e., the bacteria did not properly colonize the plant and/or compete with native microbiota). *SEMIA* 4085 succeeded in controlling collar rot disease, despite being selected as a poor in vitro inhibitor. Dual culture screens could not detect microorganisms that were effective *in planta* primarily/only through "indirect antagonism," such as inducing host resistance or competing for ecological plant niches (Knudsen et al. 1997; Pang et al. 2009). In fact, rhizobia were previously reported to elicit systemic resistance in plants (Elbadry et al. 2006).

Previous research has shown the efficacy of different biological agents in controlling *S. rolfsii* disease in common bean cultivated under pot conditions. Barakat et al. (2006) reported that different *Trichoderma* treatments reduced the disease indexes up to 66.8%. Gholami et al. (2014) reported that *Bacillus* and *Streptomyces* treatments reduced the disease severity up to 58.5%. Madi et al. (1997) reported 64% of disease reduction in soil infested with sclerotia treated with *Talaromyces flavus*. In the present study, in pot tests, common bean grown on *S. rolfsii*-infested soil and inoculated with *SEMIA* 4032, 4077, 4088, 4080 and 4085 presented no symptoms of stem rot and wilt caused by *S. rolfsii*. An exploratory field trial with *SEMIA* 4032, 4077, 4088, 4080, 4085, and 439 confirmed their biocontrol ability. The most efficient strains detected, *SEMIA* 439 and *SEMIA* 4088, decreased 18.3 and 14.5% of the *S. rolfsii*-promoted disease incidence, respectively. The AUDPC was estimated for each treatment to quantitative summary the disease intensity over time. *SEMIA* 439 and *SEMIA* 4088 also decreased 19.9 and 17.5% of the area under the collar root disease progress curve comparing to uninoculated controls, respectively. We speculate that a higher efficacy on field could be reached by controlling the requirement of pathogen inocula through a previous assessment of fungal quantity naturally present in the soil.

Besides the action of several antifungal molecules, the suppression of collar rot may be due to plant growth promotion and/or symbiotic efficiency, which could explain detection of all MAPA-recommended strains

as biocontrol agents, which are well known to significantly increase plant shoot and root mass (Fageria et al. 2014; Kellman et al. 2005; Korir et al. 2017). *SEMIA* 4088 (= H 12) strain is already recognized as an efficient N-fixing bacteria, thus being already allowed for commercial production of inoculants in Brazil. The data obtained in this work support that *SEMIA* 4088 can also be commercialized for biocontroling the collar rot caused by *S. rolfsii* on common bean crops.

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