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RESEARCH

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Endophytic bacteria from *Euphorbia antiquorum* L. protect *Solanum lycopersicum* L. against bacterial wilt caused by *Ralstonia solanacearum*

Diane Yimta Youmbi[1], Pierre Eke[1,2], Lanvin Rochal Kepngop Kouokap[1], Vanessa Nya Dinango[1], Gilbert Ghomsi Tamghe[1], Louise Nana Wakam[1,3] and Fabrice Fekam Boyom[1*]

Abstract

Background: Bacterial wilt caused by *Ralstonia solanacearum* (syn. *Pseudomonas solanacearum*) is the most devastating bacterial disease of tomato (*Solanum lycopersicum* L.) occurring in tropical zones with consequential substantial yield losses in production fields. Currently, microbial technology in cropping systems has directed investigations toward biological control agents (BCAs), emphasizing the use of plant endophytes, including bacteria. The present work aimed to evaluate the potential of endophytic bacteria from *Euphorbia antiquorum* L. to suppress bacterial wilt incited by *R. solanacearum* in plants.

Results: Of the 10 endophytic species screened in vitro for their antagonistic activity, 6 exhibited promising potency with minimum inhibitory concentration (MIC) values ranging from 62.50 to 7.81 µg/ml. These species also produced cell wall-degrading enzymes (amylase, protease and cellulase), ammonia, siderophores, indole acetic acid and salicylic acid. The 3 most potent species (*B. amyloliquefaciens* CBa_RA37, *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_BFL2) enhanced the germination of tomato seeds and protected young tomato plantlets from the devastating effects caused by *R. solanacearum* infection.

Conclusions: This investigation demonstrated that these endophytic bacteria from *E. antiquorum* L. endowed with the ability to improve the growth and protection of tomato plants could be further developed as potential biopesticides to help mitigating the effect of bacterial wilt in tomato farms. Further investigation will set the baseline for formulation and evaluation of their efficacy in the open field.

Keywords: *R. solanacearum*, Antagonistic bacteria, Antibiosis, Growth enhancement, Microbial technology, Biocontrol

Background

At least 10% of global food production is lost due to plant diseases caused by various microorganisms, including nematodes (Mahfouz, 2021), viruses (Chen et al. 2019), fungi (Yan et al. 2021) and bacteria (Chen et al. 2019). Species of bacteria belonging to several genera are devastating plant pathogens. Ranking among the top 5 pathogens, *Ralstonia solanacearum* (syn. *Pseudomonas solanacearum*), the second after *P. syringae*,

*Correspondence: fabrice.boyom@fulbrightmail.org

[1] Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box: 812, Yaoundé, Cameroon
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is distributed worldwide, causing diseases in more than 200 host plant species belonging to 50 different families, including important crops such as potato, eggplant, pepper, tobacco, banana and tomato (Zhu et al. 2019). In tomatoes, for instance, *R. solanacearum* infects plants via wounded roots or emerging secondary roots, colonizes xylem vessels and spreads rapidly to aerial parts of the plant through the vascular system (Vasse et al. 1995). Biofilm structures formed by the aggregation of bacterial cells prevent the free circulation of water and nutrients into the plant by clogging plant vessels via the production of exopolysaccharides, the major virulence factors (Kazusa et al. 2019). Symptoms in infected plants include browning the xylem, chlorosis, stunting and wilting. Infected plants usually die at an accelerated rate, and yield losses of up to 90% have been ascribed to this pathogen (Fanhong 2013). Currently, various strategies have been employed to fight against this plant pathogen, including plant breeding, field sanitation, crop rotation and chemicals. Although some success has been reported, each of these methods, particularly the use of agrochemicals, has severe limitations. The development of microbial resistance and hazardous impacts on the environment are few examples (Fahime and Gholam 2018).

In recent decades, the exploration of beneficial microorganisms, particularly endophytic bacteria, has gained significant attention as a viable and ecofriendly alternative (Tonial et al. 2020). Endophytic bacteria that have acquired the ability to colonize their host plant without causing any noticeable harmful effects have been reported to protect several crop plants, including eggplant (Raman et Gauri 2018), peanut (Xiaobing and Guobin 2014), pistachio (Etminani and Harighi 2018) and tomato (Fahime and Gholam 2018), against diseases caused by *R. solanacearum*. Therefore, endophytic bacteria have a great potential to be potent biocontrol agents against bacterial wilt diseases. With this rationale in mind, screening of endophytic bacteria living in tissues of *Euphorbia antiquorum* L. was previously conducted and several bacterial isolates that improved the growth of tomato plants and tolerance to drought were found (Eke et al. 2019).

In the present study, ability of these endophytic bacteria to promote growth and induce resistance in tomato plants against the causative agent of bacterial wilt *R. solanacearum* was evaluated.

Methods

Sources of antagonistic and pathogenic bacteria

The pathogenic bacterium *Ralstonia solanacearum* (Rs5) used in this study was isolated from infected tomato plants showing typical symptoms of bacterial wilt and maintained on tetrazolium chloride (TZC) agar. The 10 antagonistic bacteria (Table 1) investigated here belong to the *Bacilli* and *Lysinibacilli* genera and were previously isolated from healthy desert spurge of *Euphorbia antiquorum* L. (Eke et al. 2019).

Antagonistic activity of endophytic bacteria against R. solanacearum

Cell–cell confrontation test

Evaluation of the antagonistic potential of endophytes was performed as described by Achbani et al. (2000). In brief, single colonies of the pathogen (*R. solanacearum*) and bioagents were cultured overnight in Luria Bertani broth (LBB, Himedia) medium under constant stirring (150 rpm, 28 °C). The cultures were centrifuged (10,000 rpm, 5 min), and the pellets obtained were resuspended in saline solution (NaCl, 0.09%) to obtain inoculum solutions concentrated at 10^8 cells/mL. For the confrontation assay, a 500 µl of the inoculum suspension of *R. solanacearum* was inoculated onto LBA (Luria Bertani Agar) to obtain uniform bacterial lawns. Plates were kept to rest for 15 min, and after that, triplicate wells (Ǿ = 6 mm) were drilled in the medium in which 50 µl of each antagonist was poured. Negative control wells were filled with 50 µL of a sterile saline solution. The plates were sealed and incubated at 28 °C for 48 h. The inhibitory activity of antagonistic bacteria was materialized through a clear halo around the wells. Inhibition diameters were measured, and the activity was expressed in millimeters (mm).

Antibacterial activity of cultural filtrates and ethyl acetate extracts of selected endophytes

To evaluate the activity of extracellular bacterial metabolites, a loopful of each bioagent at log-phase was used to inoculate 50 ml sterile LBB medium in 100-ml Erlenmeyer flasks. The flasks were incubated under constant

Table 1 Accession numbers of endophytic bacteria isolated from *Euphorbia antiquorum*

Bioagent[a]	Plant part	NCBI accession
Bacillus amyloliquefaciens CBa_BFL2	Leaf	MH788970
Bacillus amyloliquefaciens CBa_RA37	Root	MH788971
Bacillus brevis CBb_RA14	Root	MH788977
Bacillus cereus CBc_LPR8	Root	MH788973
Bacillus velezensis CBv_BE1	Seed	MH788975
Bacillus amyloliquefaciens CBa_BFL1	Leaf	MH788972
Lysinibacillus CBa_LPR19	Leaf	MH788980
Lysinibacillus fusiformis CBl_LPR2	Root	MH788987
Lysinibacillus fusiformis CBl_LPR11	Leaf	MH788986
Bacillus Megaterium CBm_RR10	Root	MH788974

[a] Relevant information was retrieved from Eke et al. (2019)

stirring (150 rpm; 28 °C) for 6 days. For the preparation of cultural filtrates, a 5 ml of solution was taken from each flask every day for 6 days. All cultural filtrates were submitted to filtration through Whatman filter paper No 1 and centrifugation at 10,000 rpm for 15 min, and the final filtration was through filter membranes (Millipore, 0.22 µm) (Mohamed et al. 2020). The filtrates obtained were dried under ventilation at room temperature until a dry residue with constant weight was obtained.

To prepare crude extracts, a 20 ml of ethyl acetate solvent was introduced in each flask containing the remaining 20 ml of bacterial culture. Flasks were kept under constant agitation (200 rpm) for 48 h and separated using the liquid–liquid partition method. The organic phase (ethyl acetate fraction) was harvested and evaporated using a rotary evaporator system (BUCHI, Switzerland) to yield ethyl acetate extracts. Both crude ethyl acetate extracts and cultural filtrates free of bacterial cells were submitted to in vitro antibacterial activity testing using the Clinical Laboratory Standard Institute protocol (CLSI 2008).

Briefly, a 100 µl of sterile LBB medium was distributed in triplicate wells of 96-well microtiter plates. Then, 100 µl of stock solutions of culture filtrate and ethyl acetate extract from each bacterium were added to the first wells. After thorough mixing, a twofold serial dilution was performed to prepare concentrations ranging from 1000 to 7.812 µg/ml for crude extracts and from 4.0 to 0.031 mg/ml for culture filtrates. Thereafter, a 100 µl of *R. solanacearum* suspension at 10^8 CFU/ml was inoculated in the wells, except for the sterility control, which consisted of the medium without *R. solanacearum*. The negative control was consisted of *R. solanacearum* without inhibitor. The plates were incubated at 28 °C for 48 h. The antibacterial activity was expressed in terms of minimum inhibitory concentration (MIC), the lowest concentration exhibiting complete growth inhibition of *R. solanacearum.* The assay was performed in triplicate and repeated twice, and the results are expressed as the mean plus standard deviation (Mean ± SD).

Production of hydrolytic enzymes by bacterial antagonists

Cellulase activity

The ability of the bioagents to produce and release cellulase was assessed by inoculating a loopful of each antagonist on an M9 minimal salt medium containing 20 g cellulose and 1.2 g yeast extract per liter and supplemented with agar (Ramesh et al. 2008). The plates were incubated at 28 ± 2 °C for 8 days and flooded with aqueous Congo red solution (0.3%). The flooded plates were allowed to rest for 20 min at room temperature and washed with 15% NaCl. Clear halos formed around the colonies were signs of cellulose hydrolysis and thus cellulase production. The cellulase activity (clean halo) was measured and expressed in millimeters (mm). The experiment was performed in triplicate and repeated twice. The enzymatic activity index (EAI) was calculated as described by Ramos et al. (2014):

$$\text{EAI} = (a + b)/\text{a with } a = \text{the colony diameter and } b = \text{the halo diameter.} \quad (1)$$

Protease activity

The ability of the bioagents to synthesize protease was assessed by spread inoculation of each antagonist on skim milk agar (casein 0.5%, yeast extract 0.25%, dextrose 0.1%, skim milk powder 2.8% and agar 1.5%) and incubation for 24 h at 28 °C. The appearance of clear zones around the inoculation spot marked positive protease activity (Saran et al. 2007). The protease activity (clean halo) was measured and expressed in millimeters (mm). The experiment was performed in triplicate and repeated twice. The enzymatic activity index (EAI) was calculated as described above.

Amylase activity

For amylase production assessment, a loopful of each antagonist was inoculated on starch agar plates containing 1% starch and 2% agar, followed by incubation at 28 ± 2 °C for 48 h. After incubation, the plates were flooded with 1% iodine solution for 5 min and washed with distilled water to remove the excess dye (Mengistu and Pagadala 2017). The amylase activity (clean halo) was measured and expressed in millimeters (mm). The experiment was performed in triplicate and repeated twice. The enzymatic activity index (EAI) was also calculated as described above.

Screening of antagonistic bacteria for plant growth-promoting (PGP) traits

Assay for ammonia (NH_3) production

Freshly grown antagonists, at log-phase, were inoculated into 5 ml peptone water (10%) and incubated at 30 °C for 48 h as described by Cappuccino and Sherman (1992). After incubation, a 0.5 ml Nessler's reagent was added. After thorough mixing, the development of yellow–brown coloration indicated ammonia production. The OD readouts of the yellow–brown complex were made at 450 nm using an Infinite M200 microplate reader (TECAN). The ammonia concentration produced was determined using a standard curve of $(NH4)_2SO_4$ at concentrations ranging from 1

to 10 μmol/mL ($R^2 = 0.96$). The experiment was performed in triplicate and repeated twice.

Assay for phosphate solubilization

The ability of the beneficial bacterial species to solubilize inorganic phosphate was tested on Pikovskaya's agar medium, as described by Katzenlson and Bose (1959). After 3 days of incubation at 30 °C, a clear halo around bacterial colonies was indicative of phosphate solubilization. The phosphate solubilization activity (clean halo) was measured and expressed in millimeters (mm). The experiment was performed in triplicate and repeated twice.

Evaluation of siderophores production

Carboxylic siderophores

A loopful of the bacterial antagonist was inoculated in 5 ml sterile LBB and incubated under orbital shaking (150 rpm, 25 °C) for 18 h. The slurry was filtered (Millipore, 0.22 μm), 400 μl acetate buffer and 200 μl Shenker reagent (1 ml of copper sulfate $CuSO_4$ 250 μM) were added to a 200 μl aliquot of each supernatant. A decrease in the blue cuprous complex developed by carboxylic siderophores was monitored by OD readout at 280 nm using the Infinite M200 microplate reader (TECAN) (Schwyn and Neilands 1987). Siderophore production was determined using the following formula (Payne 1994):

$$(A_{\text{ref}} - As/A_{\text{ref}}) \times 100 \qquad (2)$$

where A_{ref} is the absorbance of the reference (medium) and As is the absorbance of the test sample**.**

Hydroxamate and catecholate siderophores

For hydroxamate and catecholate siderophore determination, 1 ml ferric chloride solution (2%) was added to the 18-h culture filtrate prepared above. The color change from red to purple indicated hydroxamate and catecholate siderophore production. Both hydroxamate and catecholate were quantified spectrophotometrically (Infinite M200, TECAN) at 450 nm and 495 nm, respectively (Neilands 1982). The formula mentioned above was used to determine siderophore production, expressed as a percentage (Payne 1994).

Evaluation of the phytohormones production

Indole acetic acid (IAA) production

The ability of the bioagents to produce IAA was assessed as described by Goswani et al. (2013). Briefly, the bacterial species were grown for 3 days in LBB tubes supplemented with 0.5% glucose and 500 μg/ml L-tryptophan. Then, a 5 ml of each culture was centrifuged at 9000 g for 20 min, and a 2 ml of Salkowski's reagent (2% 0.5 M FeCl3 in 35% perchloric acid) was added to an equal volume of supernatant. The mixture was then incubated in darkness for 25 min. The pink coloration developed was measured at 530 nm (Infinite M200, TECAN), and IAA was quantified by extrapolation on the IAA standard curve prepared at concentrations ranging from 10 to 100 μg/ml.

Evaluation of salicylic acid production (SA)

Salicylic acid (SA) is a plant immune response regulator; its synthesis is often triggered in response to pathogen attacks. The ability of the bacterial antagonists to produce SA was investigated following the protocol described by Meyer et al. (1992). Ten microliters of bacterial suspension at 1.5×10^8 CFU/ml was inoculated in a 5 ml of succinate medium and incubated for 48 h at 30 °C under constant stirring (100 rpm). The suspensions were centrifuged (6000 g; 5 min), and a 4 ml aliquot of the supernatants was acidified (HCl, 1 N). Salicylic acid was then extracted by liquid–liquid partitioning using chloroform, and the organic solvent was evaporated using a rotary evaporator system (BUCHI, Switzerland). The absorbance readout of the purple iron–salicylic acid complex developed upon 5 μl ferric chloride ($FeCl_3$) in chloroform extraction was made at 527 nm using a microtiter plate reader (Infinite M200, TECAN). The content of SA was determined against a standard calibration curve of pure SA ($R^2 = 0.96$) and expressed in μg/ml.

Evaluation of the in planta growth response of primed tomato plantlets

Tomato seed biopriming assay

Healthy tomato seeds (RIO Grande TM328) with no cracks or any visible deformation were selected, surface sterilized with 3% sodium hypochlorite (NaOCl) for 10 min and rinsed thrice with sterile distilled water. Air-dried seeds under the hood were seeded in Petri plates provided with sterile wet tissue paper. Antagonistic bacterial suspensions were prepared at 0.5×10^8 CFU/ml, and 5 ml inoculum of each antagonist was poured into the plates (Devi and Kumar 2020). For the control, the inoculum was replaced with sterilized distilled water. For each treatment, 5 plates of 20 seeds each were prepared. Germinated seeds were counted daily for 15 days. The germination rate (GR) and germination index (GI) were calculated:

$$\text{GR} = (\Sigma\text{DGR/N}) \times \text{GP}/10 \quad \text{(Eke et al. 2019).} \qquad (3)$$

GR = germination rate, GP = germination percentage at the end of the test, DGR = daily germination rate, N = number of daily counts

$$\mathrm{GI} = \left[\frac{\text{No of germinated seeds}}{\text{Days of first count}}\right] + \cdots + \left[\frac{\text{No of germinated seeds}}{\text{Days of last count}}\right] \quad \text{(Djavanshir and Pourbeik 1976).} \tag{4}$$

Evaluation of the biological control potential of selected endophytic bacteria against Ralstonia solanacearum

Experiments were conducted in a greenhouse to evaluate the potential of selected bacteria (BFL2, RA37 and BE1) to protect plants from *R. solanacearum*. Seeds were treated with a suspension of each biological control agent concentrated at 0.5×10^8 CFU, while control seeds were treated with distilled water. All treated seeds were germinated and grown in potting soil for 3 weeks before transplanting. Seedlings were carefully uprooted and rinsed with sterile distilled water. Root tips of the plantlets were then excised with sterile scissors and dipped in the previously prepared pathogen suspension at a concentration of 10^8 CFU for 30 min (Ji et al. 2014). Seedlings were transferred to pots filled with 300 g soil infested by *R. solanacearum* (5 mL per pot) to a final density of 1.10^8 CFU in soil. The treatments applied were codified as follows: (1) Control: uninoculated seedlings, (2) Rs5: seedlings infected with *R. solanacearum* Rs5 alone, (3) BFL2_Rs5: seedlings primed by *B. amyloliquefaciens* CBa_BFL2 and infected with *R. solanacearum* Rs5, (4) BE1_Rs5: seedlings primed by *B. velezensis* CBv_BE1 and infected *R. solanacearum* Rs5, and (5) RA37_Rs5: seedlings primed with *B. amyloliquefaciens* CBa_RA37 and infected with *R. solanacearum* Rs5. The test pots were arranged in the controlled greenhouse, following a randomized complete block design (RCBD) with 10 replications per treatment. The development of wilting symptoms was observed daily for 30 days, tomato plants were subsequently harvested, and disease parameters were evaluated.

Indeed, the percentage of wilted plants (PWP) was recorded per treatment every week after sowing. Likewise, the wilting severity (WS) for each treatment was further calculated as per the 1–5 disease rating scale reported by He (1983) as follows: (1) no visible wilting symptom on the leaf system, (2) one wilted leaf, (3) 2–3 wilted leaves with no yellowing, (4) 4 or more wilted leaves with slight leaf chlorosis and (5) broad leaf wilted or entire plant dies. The WS was therefore calculated using the formula below. The area under the disease progress curve (AUDPC) was also calculated as a function of time *f*(*tk*) and thus evaluated at a cumulative count of each single recording period (*tk*) (Forbes et al 2014).

$$\mathrm{WS}\,(\%) = [\Sigma(ni \times vi)/(V \times N)] \times 100. \tag{5}$$

where n_i = number of plants displaying similar disease severity score; *vi* given disease score in relation to *ni*; N = total number of examined plants per treatment; and V = highest wilting severity (1–5).

$$\mathrm{AUDPC} = \sum_{i=1}^{n-1} \left[\frac{y_i + y_{i+1}}{2}\right][t_{i+1} - t_i] \tag{6}$$

where t is the time of each reading; y is the percentage of affected foliage at each reading; and n is the number of readings.

Twenty-eight days after transplantation, plantlets were carefully uprooted by immersing the pots in 20 L of water bucket as a means to prevent root damage. The above- and underground parts were separated, and the root and shoot lengths were measured and expressed in centimeters (cm). The plant parts were then dried, and dry masses of the above and underground parts were recorded.

Data analyses

Data were normalized and subjected to analysis of variance (ANOVA). The generated mean values were pairwise compared using the Newman–Keuls post hoc test. The Pearson test was used to study the relationship between parameters when needed. The Sigma plot 11.0 statistical package was used for the analyses, and the significance threshold of the overall analyses was set at 5%.

Results

Cell–cell confrontation of endophytic bacteria versus R. solanacearum

Among the 10 endophytic bacteria screened, 6 species, *B. amyloliquefaciens* CBa_RA37, *B. velezensis* CBv_BE1, *B. amyloliquefaciens* CBa_BFL2, *Lysinibacillus* CBa_LPR19, *B. Megaterium* CBm_RR10 and *B. amyloliquefaciens* CBa_BFL1, inhibited the growth of *R. solanacearum* with inhibition diameters of 41, 35, 38, 43, 15 and 26 mm, respectively (Fig. 1). The species LPR2, LPR8, LPR11 and RA14 were inactive.

Antibacterial potential of endophytic bacterial cultural filtrate and extracts against R. solanacearum

The potential of cultural filtrates from the promising antagonistic bacteria against *R. solanacearum* pathogen was assessed at different culture durations to select the appropriate culture time required for the endophytes to elicit optimal levels of antibacterial potential (Fig. 2). Extracts obtained between 24 and 72 h exhibited low

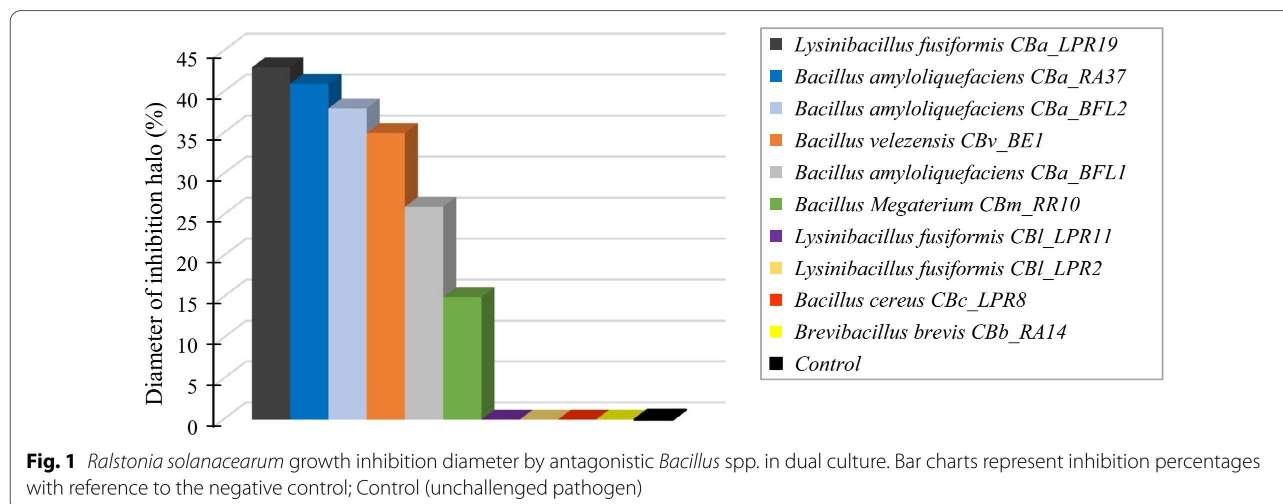

Fig. 1 *Ralstonia solanacearum* growth inhibition diameter by antagonistic *Bacillus* spp. in dual culture. Bar charts represent inhibition percentages with reference to the negative control; Control (unchallenged pathogen)

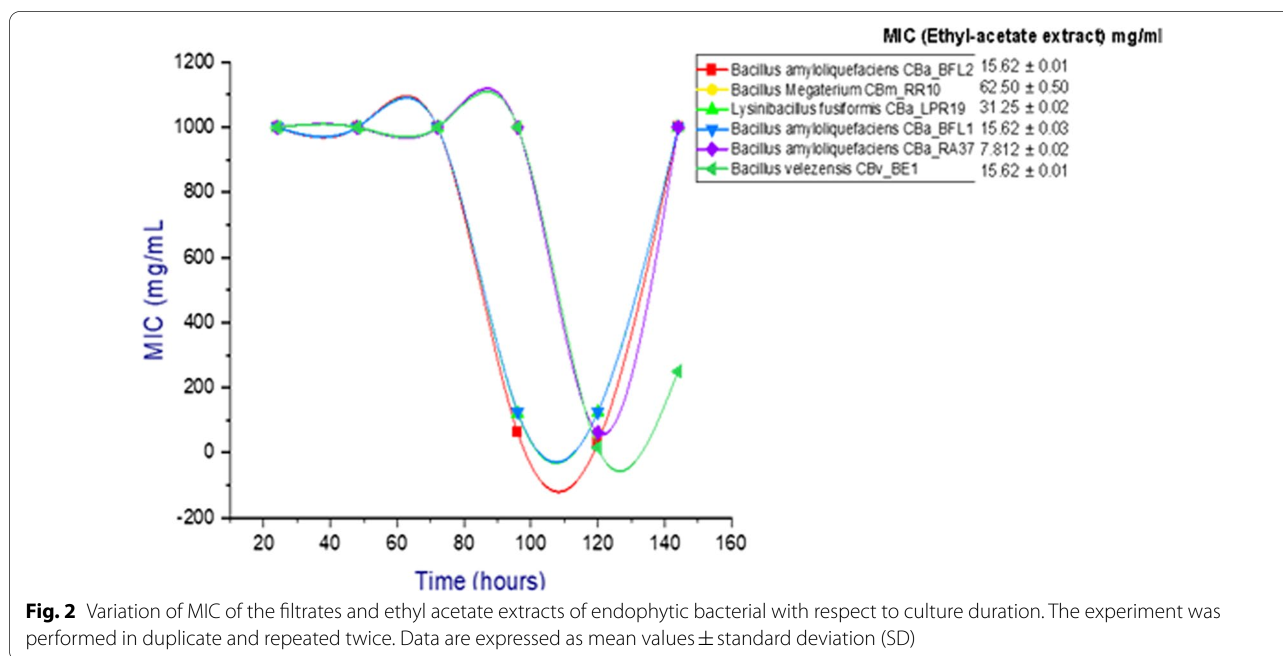

Fig. 2 Variation of MIC of the filtrates and ethyl acetate extracts of endophytic bacterial with respect to culture duration. The experiment was performed in duplicate and repeated twice. Data are expressed as mean values ± standard deviation (SD)

antibacterial potency (MIC > 1000 to 1000 μg/ml). Significantly promising activities were recorded among 96–120 with MIC values ranging from 15.62 to 1000 μg/ml). The most active endophytic extracts were obtained from *B. velezensis* CBv_ BE1 (MIC of 15.625 μg/ml) and *B. amyloliquefaciens* CBa_BFL2 (MIC of 31.25 μg/ml) upon 120 h culture. Above 120 h culture duration, a dramatic activity loss was recorded, culminating at as high as 16 to 32-fold activity drop for extracts from *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_BFL2, and a complete loss of potency for the remaining extracts. Therefore, 120 h was selected as the appropriate duration for optimal production of antibacterial metabolites in culture by selected endophytic species. Thus, the ethyl acetate extracts were prepared upon 120 h culture, and MIC values ranging from 7.812 to 62.50 μg/ml were obtained (Fig. 2). The extract from *B. amyloliquefaciens* CBa_RA37 (MIC = 7.812 μg/ml) was the most active, followed by those from *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_BFL1 (MIC = 15.625 μg/ml). Of note, the ethyl acetate extracts were significantly more active than the cultural filtrates, regardless of the bacterium species. This could be due to the ability of ethyl acetate to solubilize and concentrate the active principles from the cultural filtrates.

Table 2 Production of selected hydrolytic enzymes by endophytic bacteria

Bacterial species	Enzymatic index[a]		
	Protease production	Amylase production	Cellulase production
Bacillus velezensis CBv_BE1	1.54 ± 0.01	2.33 ± 001	2.50 ± 0.01
Bacillus amyloliquefaciens CBa_BFL2	1.72 ± 0.01	1.89 ± 0.00	2.00 ± 0.01
Bacillus amyloliquefaciens CBa_BFL1	1.33 ± 0.04	1.92 ± 0.02	1.55 ± 0.03
Bacillus amyloliquefaciens CBa_RA37	1.41 ± 0.01	2.16 ± 0.01	2.11 ± 0.02
Bacillus Megaterium CBm_RR10	1.00 ± 0.03	1.29 ± 0.01	1.06 ± 0.04
Lisinibacillus fusiformis CBa_LPR19	1.15 ± 0.06	1.43 ± 0.02	1.15 ± 0.02

[a] Stands for mean diameter of halo zones with reference to the bacterial colony diameter

Hydrolytic enzymes

The ability of selected bacteria to produce hydrolytic enzymes was investigated (Table 2). The results showed that all 6 biocontrol agents could synthesize and release amylase, cellulase and proteases with activity-induced halo zones ranging from 1.00 to 2.50 mm. Overall, *Bacillus* spp., *B. amyloliquefaciens* CBa_BFL2 (Ø1.72–1.89–2.00 mm) and *B. velezensis* CBv_BE1 (Ø1.54–2.33–2.50 mm) exerted the best proteolytic, amylasic and cellulasic activities, respectively, followed in a random order by other *Bacillus* spp., BFL1, RA37 and RR10 and *Lysinibacillus* sp., LPR19 (Ø1.00–1.92 mm).

Ammonia, siderophores and phosphate solubilization

The selected antagonistic bacteria were also evaluated for their growth promotion properties. The features, including the ability to solubilize phosphate and produce ammonia, siderophores and phytohormones, were investigated. Four out of the 6 investigated endophytic bacteria significantly solubilized phosphate, inducing halo zones ranging from 1.5 to 6.0 mm, including *B. velezensis* CBv_BE1 (6.0 mm), *B. amyloliquefaciens* CBa_RA37 (4.0 mm), *B. amyloliquefaciens* CBa_BFL2 (3.0 mm) and *B. megaterium* CBm_RR10 (1.5 mm) (Table 3). All 6 biocontrol agents produced ammonia at concentrations ranging from 0.568 to 0.829 µmol/ml. The best ammonia-producing species was *B. amyloliquefaciens* CBa_RA37 (0.829 µmol/ml), followed by *B. velezensis* CBv_BE1 (0.761 µmol/mL) and *B. amyloliquefaciens* CBa_BFL2 (0.751 µmol/ml). On the other hand, all 6 antagonistic agents produced the 3 types of siderophores, catecholate, hydroxamate and carboxylic (Table 3). *B. velezensis* CBv_BE1, *B. amyloliquefaciens* CBa-BFL2 and *B. amyloliquefaciens* CBa-RA37 produced the highest amount (100%) of hydroxamate- and catecholate-type siderophores, whereas for the carboxylic siderophores, *B.s velezensis* CBv_BE1 was by far the best producer (69.13%).

Phytohormone-producing ability of the biocontrol agents

The ability of selected endophytic bacteria to produce phytohormones, including indole acetic acid (IAA) and salicylic acid (SA), was determined (Fig. 3). The results indicated that 5 out of the 6 BCAs produced SA, with *B. velezensis* CBv_BE1 (29.53 µg/ml), followed by *B. amyloliquefaciens* CBa_RA37 (26.98 µg/ml), which was the most active. Regarding their ability to produce

Table 3 Quantitative estimation of phosphate solubilization, ammonia and siderophore production

Bacterial species	Phosphate solubilization (mm)[a]	Ammonia (µmol/ml)[b]	Siderophores (%)[c]		
			Catecholate	Hydroxamate	carboxylic
Bacillus velezensis CBv_BE1	6.0	0.761	100	100	63.13
Bacillus amyloliquefaciens CBa_BFL2	3.0	0.751	100	100	27.96
Bacillus amyloliquefaciens CBa_BFL1	0.0	0.71	89.75	77.85	6.22
Bacillus amyloliquefaciens CBa_RA37	4.0	0.814	100	100	20.20
Lysinibacillus fusiformis CBa_LPR19	0.0	0.532	44.58	61.02	7.18
Bacillus Megaterium CBm_RR10	1.5	0.607	55.43	44.84	19.26

[a] Mean diameter of clear halo around the bacterium colony

[b] Relative ammonia production in water peptone medium

[c] Percent production of each siderophore type relative to the control treatment

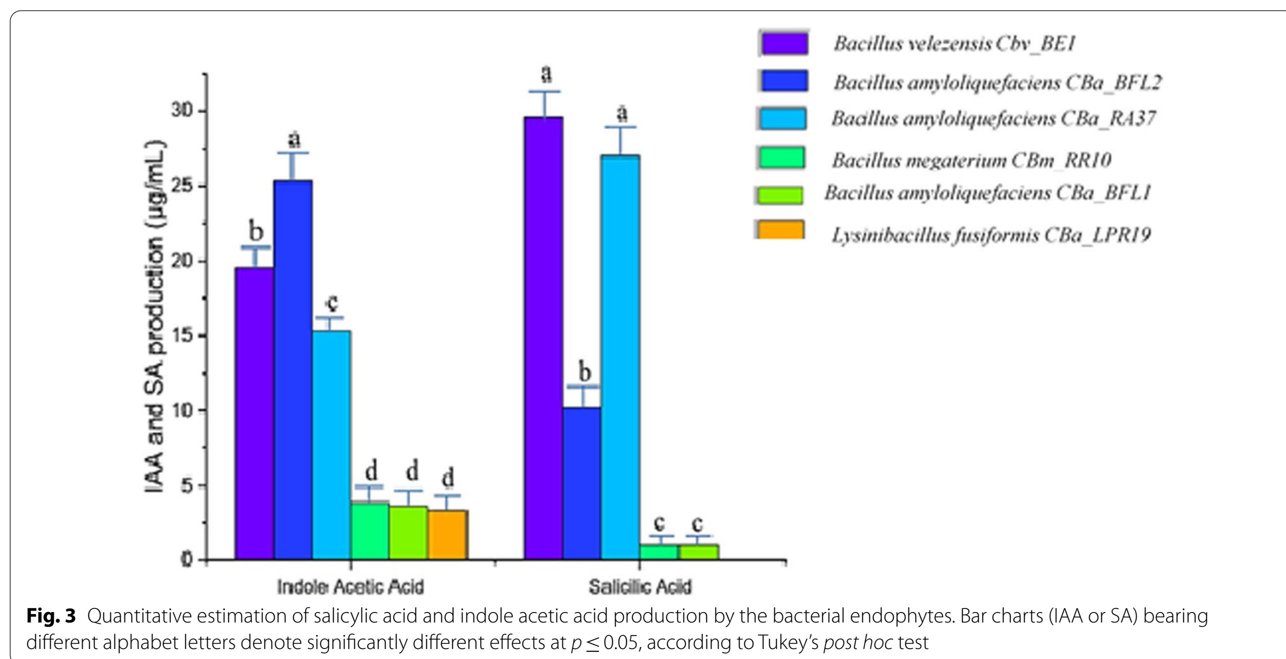

Fig. 3 Quantitative estimation of salicylic acid and indole acetic acid production by the bacterial endophytes. Bar charts (IAA or SA) bearing different alphabet letters denote significantly different effects at $p \leq 0.05$, according to Tukey's *post hoc* test

Table 4 General ranking of endophytic bacteria based on their antagonist properties against *Ralstonia solanacearum* and their plant growth-promoting traits

Code	BCA	Antagonistic activity							PGP traits				Total	Rank
		DC	EX	CF	CE	P	A	IAA	SA	PS	NH3	Sd		
MH788975	CBv_BE1	4	3	3	3	2	3	3	3	5	3	4	36	1
MH788971	CBa_RA37	5	4	1	2	2	3	3	2	3	4	4	33	2
MH788970	CBa_BFL2	4	3	2	3	2	2	2	2	3	2	4	29	3
	CBa_LPR19	5	2	1	1	1	1	2	0	0	1	2	16	4
MH788974	CBm_RR10	2	1	1	1	1	1	2	1	1	1	2	14	5
MH788972	CBa_BFL1	2	1	1	1	1	2	1	1	0	1	2	13	6
MH788987	CBl_LPR2	0	0	0	0	0	0	1	0	1	2	1	5	7
MH788977	CBb_RA14	0	0	0	0	0	0	2	0	0	2	1	5	8
MH788973	CBc_LPR8	0	0	0	0	0	0	1	1	0	1	2	5	9
MH788977	CBl_LPR11	0	0	0	0	0	0	1	0	0	1	1	3	10

BCA, biocontrol agent; DC, dual culture; EX, ethyl acetate extract; CF, culture filtrate; CE, cellulase activity; P, proteolytic activity; A, amylase activity; IAA, indole acetic acid; SA, salicylic acid; PS, phosphate solubilization; NH3, ammonia production; Sd, siderophore production

IAA, *B. velezensis* CBv_BE1 was by far the best producer (25.32 µg/ml).

A scoring value was attributed to all activities determined, and all the species investigated were classified based on individual sum of activity scores. Thus, the classification of the antibacterial and plant growth promotion activities led to the identification of 3 potent bacteria, including *B. velezensis* CBv-BE1, which was the most potent, followed by *B. amyloliquefaciens* CBa-RA37 and *B. amyloliquefaciens* CBa-BFL2, with activity scoring values of 36, 33 and 29, respectively (Table 4).

Table 5 Induced germination index and germination rate of potent biocontrol agents CBv_BE1, CBa_RA37, CBa_BFL2)

Biological control agents	GR[a]	GI[b]
Bacillus amyloliquefaciens CBa_BFL2	107.33	15.10
Bacillus amyloliquefaciens CBa_RA37	80.7179	14.16
Bacillus velezensis CBv_BE1	114.59	16.03
Control	33.694	8.81

[a] GR = germination rate

[b] Germination index; Control = Mock treatment (uninoculated seed)

Seed germination mediated by selected endophytic bacteria (CBv_BE1, CBa_RA37, CBa_BFL2)

Three of the best antagonistic agents (Table 4) exhibiting interesting growth promotion properties were evaluated in vitro for their ability to stimulate the germination of tomato seeds (Table 5). Compared to the germination index (GI) and germination rate (GR) of the control (Gr/Gi 33.69/8.81), all 3 bacteria exhibited better potency, with *B. velezensis* CBv-BE1 (Gr/Gi 114.59/16.03) being the best, followed by *B. amyloliquefaciens* CBa-BFL2 (Gr/Gi 107.33/15.1) and *B. amyloliquefaciens* CBa-RA37 (Gr/Gi 89.6/14.16). These 3 bacteria were further submitted to *in planta* studies to investigate their ability to protect tomato plants from *R. solanacearum* deterrent effects.

Bacterial wilt suppression by promising biocontrol agents under greenhouse conditions

Impact of seedling bacterization on wilt incidence, severity and tomato growth parameters under R. solanacearum Rs5 challenge

The three *Bacillus* species (CBv_BE1, CBa_RA37 and CBa_BFL2) were tested singly for their ability to dwarf off the destructive effects of the highly pathogenic *R. solanacearum* Rs5 in tomato seedlings under greenhouse conditions. The results indicated that, when plants were challenged with bacterial antagonists, disease establishment and progression dropped significantly ($p < 0.01$) compared to plants emerging from pots infected with the pathogen alone (Rs5-treated plants). The PWP (percentage of wilting plants) was ranged from 100 to 20%, with the treatment using *B. amyloliquefaciens* CBa_RA37 being the most protective with a PWP of 20% by 28th day post-inoculation (dpi), followed by *B. amyloliquefaciens* CBa_BFL2 and *B. velezensis* CBv_BE1 (PWP = 30), when compared to the PWP (100%) of the negative control (plant infected with *R. solanacearum* alone) (Fig. 4). In the case of successful infection, the wilting severity (WS) was significantly dropped by the 30th dpi, when the pathogen was challenged with the bioagents, culminating in 96, 90 and 86% protection with *B. amyloliquefaciens* CBa_RA37, *B. amyloliquefaciens* CBa_BFL2 and *B. velezensis* CBv_BE1, respectively. The growth performance of bacteria-primed tomato seedlings was assessed

Fig. 4 Biological control of bacterial wilt and enhanced growth performance in tomato seedlings under greenhouse conditions using selected *Bacillus* antagonists. The parameters were determined until 30 dpi (day post-inoculation). **a** Uninoculated seedlings (control) neither by *Bacillus* spp nor *Ralstonia solanacearum* Rs5. **b** *Ralstonia solanacearum* Rs5-infected plants without an antagonist. **c** *Ralstonia solanacearum* Rs5-infected plants primed with *Bacillusvelezensis* CBv_BE1 alone. **d** Plantlets primed with *Bacillus amyloliquefaciens* CBa_BFL2 alone and grown on *Rasltonia solanacearum* Rs5-infected soil. **e** Plantlets primed with *Bacillus amyloliquefaciens* CBa_RA37 alone and grown on *Ralstonia solanacearum* Rs5-infected soil

Table 6 BCA-primed tomato growth parameters challenged with *Ralstonia solanacearum* Rs5 pathogen

Treatment	Agro-morphological parameters, AUDPC and PWP				AUDPC	PWP
	SL (cm ± SD)	RL (cm ± SD)	RW (g ± SD)	SW (g ± SD)		
Control	19.40 ± 0.30[b]	11.50 ± 0.80[bc]	0.50 ± 0.10[b]	0.82 ± 0.20[b]	0	0
Pathogen (Rs5)	3.10 ± 0.40[a]	6.70 ± 0.60[a]	0.07 ± 0.01[a]	0.62 ± 0.10[a]	697	100
CBa_RA37 + Rs5	29.40 ± 0.60[c]	16.70 ± 0.50[c]	0.48 ± 0.10[b]	2.20 ± 0.22[d]	43	20
CBa_BFL2 + Rs5	28.60 ± 1.20[b]	16.70 ± 1.30[b]	0.94 ± 0.14[d]	2.00 ± 1.10[c]	155	30
CBv_BE1 + Rs5	26.20 ± 0.20[b]	18.10 ± 0.80[c]	0.91 ± 0.10[d]	2.20 ± 0.40[d]	135	30

SL, shoot length; RL, root length; RW, root weight; SW, shoot weight; AUDP, area under the disease progress curve; PWP, percentage of wilt plant; control, Uninoculated seedlings; Rs5, *R. solanacearum* Rs5 infected plants without antagonist; CBa_RA37 + Rs5, plantlets primed with *Bacillus amyloliquefaciens* CBa_RA37 alone and grown on *R. solanacearum* Rs5 infected soil; CBa_BFL2 + Rs5, plantlets primed with *B. velezensis* CBv_BE1 alone and grown on *R solanacearum* Rs5 infected soil; CBv_BE1 + Rs5, plantlets primed with *Bacillus velezensis* CBv_BE1 alone and grown on *R. solanacearum* Rs5 infected soil; SD, standard deviation

Mean values within each column superscripted by the same letter (a, b, c, d, or e) are not significantly different ($P < 0.01$) as given by the Turkey's HSD post hoc test

under *R. solanacearum* Rs5 challenge (Table 6). It is noteworthy that *R. solanacearum* Rs5 infestation resulted in a drastic reduction in overall growth parameters with up to 66 and 86% reductions in shoot and dry root matter, respectively, compared to unstressed (uninfected and unprimed control) plants. Interestingly, plant priming with biocontrol agents significantly enhanced seedling vigor by 157–392% relative to uninoculated seedlings. Binary priming with *B. amyloliquefaciens* CBa_BFL2 and CBa_RA37 performed far better than the other BCAs by inducing up to 229%, 392%, 157% and 319% increases in shoot length, root length, and root and shoot dry weights, respectively (Fig. 4).

Area under the disease progress curve (AUDPC)

To quantitatively summarize the disease intensity of the overall treatment over the experimental period, AUDPC was calculated (Eq. 6) by the trapezoidal integration method. This parameter transformed the disease intensity under each treatment into a measurable area across the entire experimental period. The results (Table 6) revealed that the AUDPC ranged from 43 to 697. The inoculation of the seedlings with CBa_RA37 exerted the best cumulative disease suppression potential over time (AUDPC = 43) compared to plants treated with Rs5 alone (AUDPC = 697).

Discussion

Biological control to manage plant diseases is suitable for sustainable agriculture and represents a safer alternative approach to chemical pesticides (Tahira and Saad, 2020). In this regard, there is a growing interest in endophytic bacteria as potential biocontrol agents due to their multiple properties, including plant growth promotion, antagonistic effects on pests and pathogens, alleviation of abiotic stressors and phytoremediation (Mercado-Blanco and Lugtenberg 2014). Therefore, endophytic bacteria from either the same crop (Feng et al. 2013) or unrelated crops (Thomas and Upreti 2014) reported their biocontrol efficiency against bacterial wilt in tomatoes. Abilities of endophytic bacteria from *E. antiquorum* L. to promote the growth of tomato plants and supress wilting symptoms caused by *R. solanacearum* in tomato plantlets under greenhouse conditions were investigated.

Upon screening for antagonistic potential, 6 out of 10 endophytic bacteria displayed superior antagonistic effects toward *R. solanacearum* in vitro via the production of diffusible extracellular bioactive metabolites. It is well established that the in vitro secretome of endophytic bacteria may contain a wide range of molecules, such as specialized secondary metabolites and enzymes with antibacterial activity (Pereira et al. 2016). The fact that all the potent species identified in this work belonged to the *Bacillus* genus is not surprising. Indeed, the potential of *Bacillus* species to produce a wide diversity of bioactive secondary metabolites (an estimated 795 secondary metabolites to date), such as antibiotics, cyclic lipopeptides, polyketides (PKs) and bacteriocins, has been recognized for decades (Horak et al. 2019). Obtained data clearly indicated a time-dependent secretion of antimicrobial metabolites by the *Bacillus* species. Notably, highest activity (low MICs) indicated that more active metabolites were secreted after 120 h of culture, irrespective of the bioagent. A similar profile was reported by Strobel et al. (2008), with the fungus *Gliocladium roseum*, which produced promising antimicrobial metabolites upon 18 days of incubation. These results were thereafter ascribed to the nutrients scarcity into the culture medium and the microbial saturation, leading to a stressful condition triggering the defensive mechanism of bioagents.

Out of the 6 antagonists reported in this work, 3, namely; *B. amyloliquefaciens* CBa_RA37, *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_BFL2,

displayed plant growth promotion properties. A previous screening of 10 bioagents by Abo-Elyousr et al. (2019) led to the identification of *B. subtilis* and *B. amyloliquefaciens* as the most potent against the causative agent of tomato wilt. Interestingly, the 3 *Bacillus* species identified in the present study were able to improve the germination of tomato seeds in vitro and enhance the growth of the plants in greenhouse conditions. Plants treated with the *Bacillus* species were more vigorous, and their growth was significantly increased regardless of inoculation with the *R. solanacearum* pathogen. Similarly, Abo-Elyousr et al. (2019) reported the growth improvement of tomato plant inoculated with a species of *B. amyloliquefaciens*. The induced increase in root growth may be attributed to bacteria's ability to improve plant phosphate solubilization and the synthesis of plant growth-regulating substances such as IAA and siderophores (Antunes et al. 2017). Indeed, phosphorous nutrition enhances the overall growth of the plants and helps in root development (Jones and Darrah 1994), and increasing IAA production may positively influence the development of the root system and allow the plants to improve the nutrient uptake that is critical for their growth. Nutrient acquisition could also be enhanced through siderophore production, which is known to increase the bioavailability of nutrients such as iron (Glick 2003).

In addition to their ability to improve the growth of tomato plants, the 3 *Bacillus* species significantly reduced the severity of bacterial wilt disease caused by *R. solanacearum*. The reduction in wilt incidence and severity in infected plants could be due to the capacity of these biological control agents to produce specialized antibiotic metabolites that inhibit the growth of *R. solanacearum*. The biocontrol ability of bacteria from the genus, *Bacillus* against multiple plant pathogens was very well documented (Abo-Elyousr et al., 2019), emphasizing interest in exploiting their biofertilizer or biopesticide beneficial traits in agriculture (Li et al. 2019). As for internal tissue colonizers, they could directly provide a barrier against invading pathogens and improve plant resistance by competing for space or nutrients with pathogens and by producing bioactive substances and cell wall-degrading compounds (Abo-Elyousr and Hassan 2021). These traits could further explain the beneficial effect against bacterial wilt that was demonstrated in the present study. Overall, *B. amyloliquefaciens* CBa_RA37, *B. velezensis* CBv_BE1 and *B. amyloliquefaciens* CBa_BFL2 exhibited both growth promotion and disease protection in tomato plants. The 3 biocontrol agents can produce remarkably resistant spores to resist environmental challenges, colonize the plant root system and inhabit plant tissues, while promoting host growth and protection (Gouda et al. 2018). This combination of beneficial traits made these 3 bacteria ideal microorganisms for further development of new biological pesticides to fight against bacterial wilt caused by *R. solanacearum.*

Conclusions

The ability of endophytic bacteria isolated from *E. antiquorum* to protect tomato crops against the pathogen *R. solanacearum* was assessed. From the 10 initial endophytic bacteria chosen for this study, 3 of the genus *Bacillus*, namely, *B. amyloliquefaciens* CBa_RA37, *B. amyloliquefaciens* CBa_BFL2 and *B. velezensis* CBv_BE1, showed promising inhibitory potentials in vitro and in vivo against the *R. solanacearum* pathogen. It was concluded that the described findings were contributed to a better understanding of the fundamental aspects of host–pathogen–endophyte interactions. They therefore open the scope for further explorations into the biological control of the *R. solanacearum* pathogen using antagonistic agents from local biodiversity.

Abbreviations

AUDPC: Area under the disease progress curve; BCA: Biological control agent; CLSI: Clinical Laboratory Standard Institute; EAI: Enzymatic Activity Index; FAO: Food and Agricultural Organization; IAA: Indole acetic acid; MIC: Minimum inhibitory concentration; ONU: Organization of United Nations; PWP: Percentage of wilted plants; SA: Salicylic acid; SD: Standard deviation; TZC: Tetrazolium chloride; WS: Wilting severity.

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Author contributions

LN and FB contributed to the study conception and design. DY, EP, LK, VN and GG performed material preparation, data collection and analysis. The first draft of the manuscript was written by DY and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Author details
[1]Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, P.O. Box: 812, Yaoundé, Cameroon. [2]Department of Crop Production Technology, College of Technology, University of Bamenda, P.O. Box 39, Bambili, North West Region, Cameroon. [3]Laboratory of Soil Microbiology, Department of Microbiology, Biotechnology Centre, Nkolbisson, Yaoundé, Cameroon.

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