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Research Paper

Biological control of clementine branch canker, caused by *Phytophthora citrophthora*

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Summary. Significant losses of clementine trees (*Citrus* × *clementina*) due to *Phytophthora* branch canker were observed in the Cap Bon Peninsula in northeastern Tunisia. This disease is caused by *Phytophthora citrophthora*. The low efficiency of available cultural and host resistance disease management methods, and potential harmful impacts of pesticide strategies, lead to a search of new control alternatives. This study investigated potential biocontrol agents. The halophilic bacterial strains *Bacillus pumilus* M3-16 and *Halomonas elongata* L80, previously selected for their antifungal activity, were assessed in a greenhouse trial. In addition, 69 endophytic bacteria were isolated from citrus roots and assessed for their antagonistic activities. Five isolates were selected because they showed strong growth inhibition of *P. citrophthora* in dual culture tests. Based on phenotypic characteristics, biochemical tests and sequence analyses of the 16S region of rDNA, the bacteria were identified as *Bacillus pumilus* (S19), *Bacillus amyloliquefaciens* (S24), *Bacillus siamensis* (S54) *Paenibacillus polymyxa* (S31), and *Pseudomonas veronii* (S40). These metabolized different carbon sources, and possessed antibiotic genes, produced siderophores and phytohormones, and solubilized phosphates. In greenhouse trials, two endophytic strains (S24, S31) and two extremophile strains (M3-16, L80) were also assessed, these bacteria significantly reduced ($P<$ 0.0001) necrotic lesions on host plants, by 35% for strain S24 of *Bacillus amyloliquefaciens* to 51% for strain L80 of *Halomonas elongata*. This is the first report of *Halomonas* sp. Controlling a *Phytophthora* sp. The strains described in this study offer a foundation for developing an efficient biofungicide.

Keywords. Branch canker, biocontrol, *Bacillus*, *Halomonas elongata*.

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INTRODUCTION

Citrus fruits are an economically important crop in Tunisia, which produces approx. 350,000 tons of citrus fruits annually (Mahjbi *et al*., 2016) on over 21,000 ha (FAO, 2013). However, intensification of cropping techniques, exchange of plant material, and competition between citrus producing countries have contributed to the emergence of disease epidemics such as Citrus

greening, Citrus canker and tristeza (Cambra*et al*., 2000; Gottwald *et al*.,2002; Bove, 2006). Among the pathogenic fungi infecting citrus, *Phytophthora* is one of the most virulent, causing damping off, root rot, brown rot, and gummosis (Graham *et al*., 2003). Although ten species have been reported to be pathogenic toward citrus trees around the world, three cause the most serious disease, stem gummosis, as well as root and fruit rot. These are *Phytophthora citrophthora*, *P. nicotianae* (syn. *P. parasitica*), and *P. palmivora* (Erwin *et al*., 1996). These three *Phytophthora* species have distinct temporal and climatic requirements, so their relative distributions and importance vary in different production areas (Matheron *et al*., 1997). *Phytophthora nicotianae* and *P. palmivora* are major causes of citrus diseases in the United States of America, whereas *P. citrophthora* predominantly causes disease in the Mediterranean Basin (Graham *et al*., 1998; Cacciola*et al*., 2008; Khanchouch *et al.,* 2017).

Clementine production has expanded in the last few years to reach second in citrus production in Tunisia, after the 'Maltaise` cultivar, and third in citrus exports (GIFruits, 2018). This progress comes after the introduction of new varieties such as 'Marisol' and 'Hernandina' via the FAO program in 1996 from Corsica, France.

Since 2015, in the Cap-Bon Peninsula in northeastern Tunisia, a region which represents 70% of the country's citrus fruit production, a new serious disease has appeared, which was found to be caused by *Phytophthora citrophthora* (Zouaoui *et al*., 2016). This syndrome was reported in Spain in 2008 (Alvarez *et al*., 2008a) and in South Africa in 2010 (Schutte *et al*., 2010), and is characterized by host gum exudation, browning of the crust, cracking, and cankers. Reasons for emergence of this disease are still unclear, but could include several hypotheses including climate change, the introduction of susceptible varieties, the appearance of a virulent strain of *P. citrophthora* (Cohen *et al*., 2003) and the roles of snails and ants as dispersal vectors of *P. citrophthora* (Alvarez *et al*., 2009).

The use of chemical pesticides is decreasing, due to potential negative side effects, including contamination of soil and ground water, health risks to humans, and development of pesticide resistance in pathogens (Conacher and Mes, 1993; Jepson *et al*., 2014). Biocontrol, particularly the suppression of plant pathogens by antagonistic bacteria, is a potential alternative.

Endophytes are plant-associated microorganisms that live in plant tissues without causing any detrimental effects to their hosts (Kloepper *et al*., 2006; Ryan *et al*., 2008). In many studies, endophytic bacteria such as *Pseudomonas* and *Bacillus* have provided effective ways to manage plant diseases, and improve plant growth (Lee *et al*., 2008; Khabbaz *et al*., 2015). These bacteria commonly produce modified or unmodified peptides, simple heterocyclic (phenazine) and aliphatic compounds, hydrogen cyanide (HCN), siderophores, volatile compounds, proteolytic enzymes such as cellulase and β-1, 3-glucanase (Susi *et al*., 2011; Radhakrishnan *et al*., 2017; Biessy and Filion, 2018). *Bacillus* spp. have several advantages such as their ubiquity, spore production, heat resistance, and antibiotic production. *Pseudomonas* spp. are excellent competitors towards soil fungal and bacterial microflora. *Pseudomonas* bacteria have the ability to use plant exudates as nutrients (Lugtenberg *et al*., 2002; Espinosa-Urgel 2004), and to produce chelating ferric ions and a wide variety of secondary metabolites, and induce systemic resistance (ISR) in plants. All these characteristics are essential for effective biocontrol.

Previous studies have shown the efficiency of halotolerant and moderately halophilic bacteria isolated from different Tunisian Sebkhas (shallow salt lakes) to control several phytopathogenic fungi, including *Fusarium sambucinum* causing dry rot of potato and gray mold in strawberry and tomato fruits (Sadfi *et al*., 2001). The halophilic bacteria are known to produce a wide range of extracellular antibiotics and volatile compounds, as well as antifungal enzymes such as protease, chitinase and glucanase (Sadfi-Zouaoui *et al*., 2008a; Essghaier *et al*., 2009b).

The objectives of the present study were to: (i) isolate and evaluate endophytic bacteria from citrus roots for control of Citrus branch canker, (ii) identify plant growth-promoting bacteria (PGPB) traits of antagonism and antibiotic and phytohormone production, and the degradation of different carbon sources, and (iii) assess the abilities of selected halolerant and endophytic bacteria to reduce development of host necrosis caused by *P. citrophthora* under greenhouse conditions.

MATERIALS AND METHODS

Isolation of citrus root endophytic bacteria

Endophytic bacteria were isolated from citrus roots. Ten different citrus cultivars, all grafted on sour orange, were randomly collected from Cap Bon Peninsula. Sampled roots were surface disinfected with 1% sodium hypochlorite solution for 10 min. The external portion of each root (approx. 5 mm from the margin) was removed with a sterile scalpel, and the root tissue was triturated in a sterile porcelain mortar in 10 mM phosphate buffer (pH 7.2). The root extracts were spread on tryptic soy agar, (TSA, Biolife) and then incubated at 28°C for 48

h. Resulting bacterial isolates were initially screened for antagonistic properties, and grouped by morphological characteristics (Haque *et al*., 2016).

Antagonistic bacteria

Two halophilic antifungal bacterial strains from Tunisian Sebkhas, *Bacillus pumilus* isolate number M3-16, and *Halomonas elongata* L80, were used in this study. The morphological, physiological, and molecular characteristics of these strains were previously described and their 16S rDNA sequences have been deposited in the GenBank database under the accession numbers EU435355 for M3-16 and EU435356 for L80 (Essghaier *et al*., 2009a).

Pathogen inoculum

The strain *Phytophthora citrophthora* (E1P1) was previously reported as the causal agent of clementine trunk and branch canker in Tunisia (Zouaoui *et al*., 2016). The ITS1/ITS4 rDNA sequences of this strain have been deposited in the GenBank database under the accession number KX269827.

Dual culture tests for antagonism

Antagonistic bacteria were selected by the co-culture test. For each test, a mycelium plug (5 mm diam.) of *P. citrophthora* was transplanted at 2.5 cm from each side of the bacterial strip located in the middle of each Petri dish. These tests were carried out on potato dextrose agar, and the incubation was for 7 d at 28°C. The percentage inhibition of *P. citrophthora* was calculated according to the formula developed by Whipps (1987): I = (R 1 -R 2) / R1 * 100. R1 was the radial distance of the pathogen colony measured from the centre to the side of the Petri plate, and R2 was the radial distance of the colony from the centre to the bacterial strip (Sadfi-Zouaoui *et al*., 2008a).

Morphological characterization of bacterium strains

The effective endophytic bacteria were phenotypically characterized according to morphological, physiological, and chemical analyses, based on the following tests: colony and cell morphology, motility, Gram staining, pigmentation, and catalase and oxidase tests (Trotel-Aziz *et al*., 2008). In addition, biochemical features of the highly antagonistic bacteria were investigated using API 20NE and ZYM strips (bioMérieux), following the manufacturer's instructions.

Molecular characterization of strains

To extract the DNA, bacteria from 24 h tryptic soy broth (TSB, Biolife) cultures were processed by centrifugation at 8000 rpm for 10 min. The bacterial pellets were then suspended in 500 µL of TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8) and treated with 17 µL of lysozyme (30 mg mL^{-1}) for 30 min at 37°C. Six µL of proteinase K (20 mg mL^{-1}) and 40 µL of 10% SDS were then added to this suspension which was incubated at 37°C. The solution was homogenized thoroughly after the addition of 100 µL of 5M NaCl and 80 µL of CTAB / NaCl (10% 0.7M) and incubated at 65°C for 10 min. An equal volume of chloroform/isoamyl alcohol (24/1) solution was then added. The aqueous phase obtained after centrifugation for 20 min at 12000 rpm was transferred to a new tube, to which 2 volumes of isopropanol was added. After incubation at -20°C for 1 h, the preparation was centrifuged for 5 min at 13000 rpm. The precipitated DNA was washed with 70% ethanol, dried and then resuspended in 50 µL of TE solution (Sambrook *et al*., 1989). The DNA obtained was stored at -20°C.

Amplification of the 16 rDNA was carried out using PCR, with the universal primers 27f (5′- AGA GTT TGA TYM TGG CTC AG-3′) and 1492r (5′-TAC CTT GTT A**Y**G ACT T-3′) (Reysenbach *et al*., 1992). The PCR profile was initial denaturation at 96°C for 3 min followed by 30 annealing cycles at 57°C for 30 s, extension at 72°C for 2 min, and denaturation at 96°C for 30 s, and an extension cycle of 72°C for 7 min. The PCR products were cleaned using the PCR purification kit (Promega) and sent for sequencing. The sequences were compared using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) for identification of the isolates. A neighbour-joining phylogenetic tree was produced by MEGA v7.0 (Kumar *et al*., 2016).

Modes of action of antagonistic bacteria

Cellulase production

Endoglucanase activity of the strains was determined as described by Miller (1959), based on the amount of sugar released during the hydrolysis of cellulose. This is determined colorimetrically, using detection by dinitrosalicylic acid 3 (DNS). The protocol for each sample was as follows: 200 µL of supernatant was added to 200 µL of the substrate 1% carboxymethyl cellulose (CMC)

and 200 µL of sodium citrate buffer (pH 4.8), then the reaction mixture was incubated at 50°C for 30 min. The reaction was stopped by the addition of 800µL of DNS. The mixture was then heated to 100°C for 15 min and then cooled in an ice bath. The nil controls each consisted of 200µL of buffer plus 800µL of DNS. Absorbance was determined at 540 nm, and converted to the concentration of reducing sugars using a standard curve created by different concentrations of glucose as standard. One unit of enzyme activity was defined as the amount of enzyme that released 1 µM of reducing sugars per 30 min (Assareh *et al*., 2012).

Siderophore production

A medium containing chromium azurol S (CAS) was used to detect the secretion of siderophores (Schwyn and Neilands, 1987). The principle is that the culture medium is initially blue, due to the iron-hexadecyl-trimethylammonium complex (iron/CAS/HDTMA complex), which turns orange when the iron is displaced by siderophore produced by the microorganism. This competition for iron will favour the siderophore forming orange ferri-siderophores. After 14 d of culture incubation at 28°C, red to orange halos form around bacterial colonies. Visual examination ensured the ability of microorganisms to produce siderophores. The strain colonies were classified for growth as + (0 to 10 mm), ++ (10 to 20 mm), or +++ (20 to 30 mm).

Phosphate solubilization

Phosphate solubilization was detected with the method of Islama *et al.* (2007), using the National Botanical Research Institute's phosphate (NBRIP) growth medium. This contained (per litre) 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 15 g agar. pH 7.0. Inoculated plates were incubated at 24±2°C. The solubilization zones around bacterial colonies were assessed 7 d after inoculation, by subtracting the diameters of bacterial colonies from the total zone diameters (dp). The strains colonies were classified as + (5 mm <dp> 15 mm), ++ (15 <dp> 20 mm), or +++ (dp>20 mm).

Indole acetic acid (IAA) production

For each sample, 100 µL of fresh culture was inoculated into 10 mL of TSB medium and incubated at 28°C for 5 d. with agitation. The cultures were each centrifuged at 10000 rpm for 5 min, 1 mL of the supernatant was then removed, 2 mL of Salkowskire agent and 100 µL of orthophosphoric acid (10 mM) were added, and the cultures were then incubated for 30 min at 25°C. Observation of pink color indicated production of IAA (Ahmad *et al*., 2008; Tarnawski *et al*., 2006). Quantity of IAA was determined by absorbance at 530 nm, converted to concentration of IAA (μgmL^{-1}) using a standard IAA curve.

PCR detection of antibiotic biosynthesis genes

The five selected bacterial strains were screened for production of lipopeptide antibiotics, using specific primers that amplify genes from fengycin (FENDF, GGCCCGTTCTCTAAATCCAT; FENDR, GTCATGCTGACGAGAGCAAA), bacillomycin (BMYBF, GAATCCCGTTGTTCTCCAAA; BMYBR, GCGGGTATTGAATGCTTGTT), bacilysin (BACF, CAGCTCATGGGAATGCTTTT; BACR, CTCGGTCCTGAAGGGACAAG), Surfactin (SRFAF, TCGGGACAGGAAGACATCAT; SRFAR, CCACTCAAACGGATAATCCTGA) and iturin (ITUD1F, GATGCGATCTCCTTGGATGT; ITUD1R, ATCGTCATGTGCTGCTTGAG). The PCR amplifications were performed in 15 µL reaction mixtures each containing 0.06 µL (5 U μL^{-1}) *Taq* DNA polymerase, 1 µL (20 ng μL^{-1}) of DNA template, 1.5 µL of 10× PCR buffer, 0.45 µL (50 mmol L^{-1}) of $MgCl_2$, 1 µL (10 mmol L^{-1}) of dNTPs, 0.3 µL (10 µmol L^{-1}) of each primer, 0.75 µL (10 mg L^{-1}) of bovine serum albumin (BSA), and 9.64 µLof filter-sterilised Milli-Q water (Mora *et al*., 2011; Khabbaz *et al*., 2015).

Biocontrol screening in planta

Pathogen inoculum

Phytophthora citrophthora (strain E1P1) was grown on V8 juice agar (JV8A) in Petri plates for sporangium production. The strain was incubated for 2 d in the dark at 24°C. The Petri plates were then filled with the Chen-Zentmyer's Salt Solution (containing, per L of distilled water: 1.64 g (Ca $(NO_3)_2$, 0.05 g KNO_3, 0.48 g $MgSO_4$, and 1 mL of chelate iron solution (13.05 g L^{-1}EDTA; 7.5 g L^{-1} KOH; 24.9 g $L^{-1}FeSO_4$; pH.7), and incubated under continuous fluorescent light at 24°C. Sporangia were produced within 1 to 2 days.

Bacterium strains

Four bacteria were selected for *in-planta* tests. Two halophilic strains (M3-16, L80) and two endophytic bacteria (S24 and S31) were used. They were grown for 48 h on TSA, supplemented with 5% NaCl for the two halo-

philic strains. After 48 h, colonies were scraped from the agar surfaces in Petri dishes, and were diluted in sterilized saline solution (1% NaCl). Bacterium concentrations were determined by dilution plating on TSA, and adjusted to 10^8 colony forming units (CFU) mL^{-1} for inoculations.

Experimental design

One hundred 2-year-old clementine plants ('Hernandina'), grafted on 'Carrizo' citrange rootstock, were grown in a partially controlled greenhouse at the Tunisian National Institute of Agricultural Research. The experiment was arranged in a completely randomized design. Five treatments were applied, each on 20 plants with two inoculation points for each plant. These were: untreated plant inoculated with *P. citrophthora* (E1P1), d, (ii) plant inoculated with antagonist *B. amyloliquefaciens* (S24) and *P. citrophthora*, (iii) plant inoculated with the antagonist *P. polymyxa* (S31) and *P. citrophthora*, (iv) plant inoculated with the halophilic antagonist *H. elongata* (L80) and *P. citrophthora*, and (v) plant inoculated with the halophilic antagonist *B. pumilus* (M3-16) and *P. citrophthora*.

Co-inoculations

For each plant, two cuts of length 5 to 10 mm were made with a scalpelon the bark of the scion, at 15 to 20 cm from each other. Each wound was immediately drop-inoculated with 100 µL of suspension of the bacterial strains (10^8 CFU mL^{-1}). The liquid was allowed to dry for 20–40 min at air temperature, and the incision was filled with a JV8A plug containing sporangia of *P. citrophthora* E1P1 placed in direct contact with the stem cambium. The wound was then wrapped with foil, moistened, and sealed with a strip of paraffin film to prevent desiccation.

Pathogenicity assessments and data analyses

The results were assessed 10 d after inoculation. Bark of the inoculated area on each plant was scraped with a scalpel, and margins of lesions were measured with a ruler. Statistical analyses for these data were performed using R statistical software version 3.4.0 (R Core Team, 2017). Normal distributions of the data were verified by the Shapiro-Wilk test before being subject to the non-parametric Kruskal-Wallis tests. Additionally, average reduction in lesion size [% reduction = 100 – (average lesion size for treatment × 100)/average lesion size for experimental control)] was calculated for the treatments that displayed statistically significant differences from the non-treated controls (Alvarez *et al.*, 2008b).

RESULTS

Isolation, characterization and identification of antagonistic bacteria

Among the 69 bacteria isolated from citrus roots, five isolates (S19, S24, S31, S40 and S54) were shown to be the most effective for inhibiting growth of *P. citrophthora* in the dual culture tests (Figure 1). Growth inhibition percentage (GI%) ranged from 67% for S31 to 74% for isolate S19. The GI% for both S24 and S54 was 69%, and for S40 was 62% (Figure 2).

Isolates S19, S24, S31, and S54 were identified as belonging to *Bacillus* and S40 to *Pseudomonas* on the basis of several phenotypic features (Table 1). The colony morphology of the *Bacillus* isolates was circular with cream pigmentation, the isolates had rod cell shape and were Gram positive, while the *Pseudomonas* isolate had pale yellow colonies, coccoid cell shape and was Gram negative (Table 1). According to API 20NE strip (bioMérieux) tests, positive reactions were observed among all the isolates for nitrate reduction, esculin and gelatin hydrolyses. Negative reactions were observed for L-tryptophane, D-glucose, L-arginine, urease, capric acid, adipic acid and phenylacetic acid. All other reactions were different among the isolates (Table 1). For

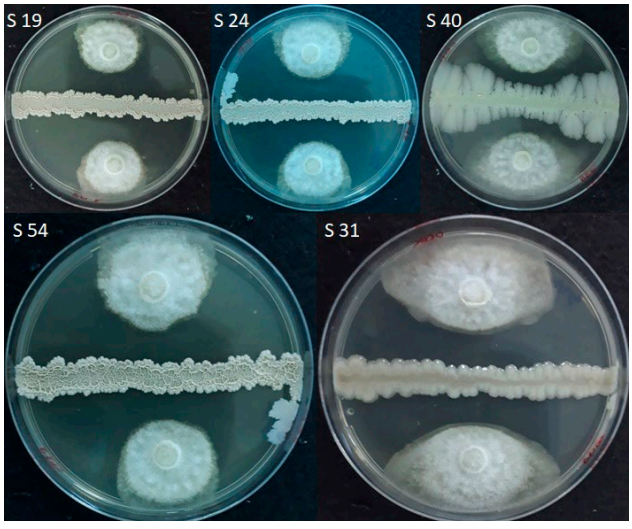

Figure 1. Dual culture showing antagonistic activities of the endophytic bacteria (strains S19, S24, S31, S40 and S54) toward *Phytophthora citrophthora*.

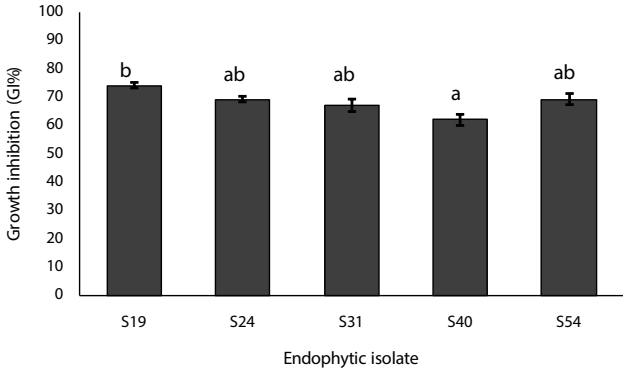

Figure 2. Growth inhibition rates of the endophytic bacteria toward *Phytophthora citrophthora*. Growth inhibition values followed by different letters are significantly different (Tukey Test, $P< 0.05$, n = 6, (± standard errors)).

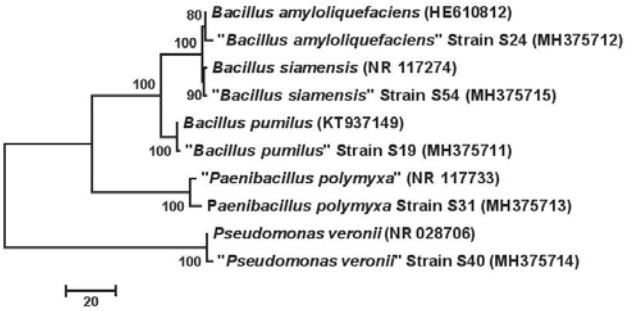

Figure 3. Neighbour-joining tree based on 16S rDNA sequences showing the phylogenetic relationship between strains S19, S24, S31, S40 and S54. Bootstrap values (expressed as percentages of 1000 replications) >70% are given at the nodes.

API ZYM strip tests, positive reactions were observed for alcaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Positive reactions for α-chymotrypsin were only observed for the isolates S31 and S40, and positive reaction for α-galactosidase occurred only for the isolate S19. All other reactions were negative (Table 1).

BLAST analyses of the 16SrDNA sequences showed that there was 99% homology of isolate S19 with *Bacillus pumilus* L14 (KT937149), isolate S24 with *Bacillus amyloliquefaciens* BD18C2-B16 (HE610812), isolate S31 with *Paenibacillus polymyxa* DSM13, S40 with *Pseudomonas veronii* CIP104663 (NR_028706), and isolate S54 with *Bacillus siamensis*PD-A10 (NR_117274). The 16S rDNA sequences were deposited in the GenBank database, under the accession numbers MH375711 for S19, MH375712 for S24, MH375713 for S31, MH375714 for S40 and MH375715 for S54 (Figure 3).

Table 1. Phenotypic characterization of selected antagonistic bacteria[a].

Characteristic	S19	S24	S31	S40	S54
Colony morphology	Circular	Circular	Circular	Circular	Circular
Cellshape	Rod	Rod	Rod	coccoid	Rod
Mobility	+	+	+	+	+
Pigmentation	Cream	Cream	Cream	Cream	Cream
Gram strain	+	+	+	-	+
Catalase	+	+	+	+	+
Oxidase	+	-	-	+	+
Nitrate reduction	+	+	+	+	+
L-tryptophane	-	-	-	-	-
D-glucose	-	-	-	-	-
L-arginine	-	-	-	-	-
Urease	-	-	-	-	-
Esculinhydrolysis	+	+	+	+	+
Gelatinhydrolysis	+	+	+	+	+
4-nitrophenyl-βD-galactopyranoside	-	-	+	-	-
L-arabinose	+	+	+	+	-
D-mannose	+	+	-	+	+
D-mannitol	+	+	-	+	+
N-acetyl-glucosamine	+	+	-	-	+
D-maltose	+	+	-	+	+
Potassium gluconate	+	-	+	+	+
Capricacid	-	-	-	-	-
Adipicacid	-	-	-	-	-
Malicacid	+	+	-	+	+
Trisodium	+	-	-	+	+
Phenylacetic	-	-	-	-	-
Alcaline phosphatase	+	+	+	+	+
Esterase (C4)	+	+	+	+	+
Lipase (C8)	+	+	+	+	+
Lipase (C 14)	-	-	-	-	-
Leucine arylamidase	-	-	-	-	-
Valine arylamidase	-	-	-	-	-
Cystine arylamidase	-	-	-	-	-
Trypsin	-	-	-	-	-
α-chymotrypsin	-	-	+	+	-
Acid phosphatase	+	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+
α-galactosidase	+	-	-	-	-
ß-galactosidase	-	-	-	-	-
ß-glucuronidase	-	-	-	-	-
α-glucosidase	-	-	-	-	-
ß-glucosidase	-	-	-	-	-
N-acetyl-ß-glucosaminidase	-	-	-	-	-
α-mannosidase	-	-	-	-	-
α-fucosidase	-	-	-	-	-

[a]S19, S24, S31 and S54: Strains of *Bacillus* sp.; S40: Strain of *Pseudomonas* sp.
(+) Positive reaction ; (-) negative reaction.

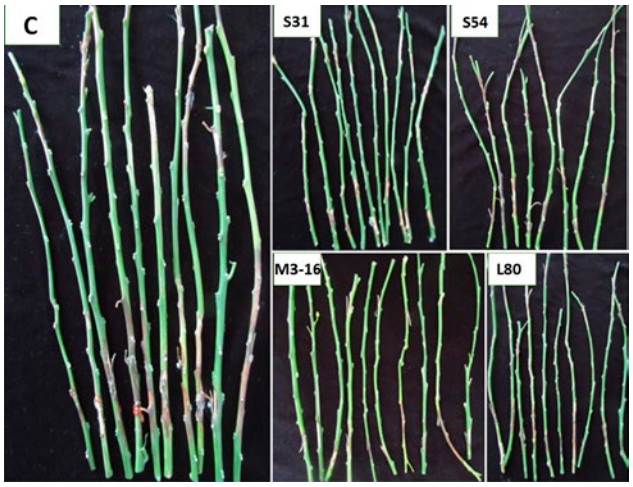

Figure 4. Suppression of branch canker on clementine mandarin for the trial conducted under partly controlled greenhouse conditions. Host branches were cut and leaves removed for improved view of the symptoms. C: Plants inoculated with *Phytophthora citrophthora* (Pc) without bacterial treatment. S31 and S24 indicate plants treated with endophytic bacteria, and M3-16 and L80 indicate those treated with halophilic bacteria.

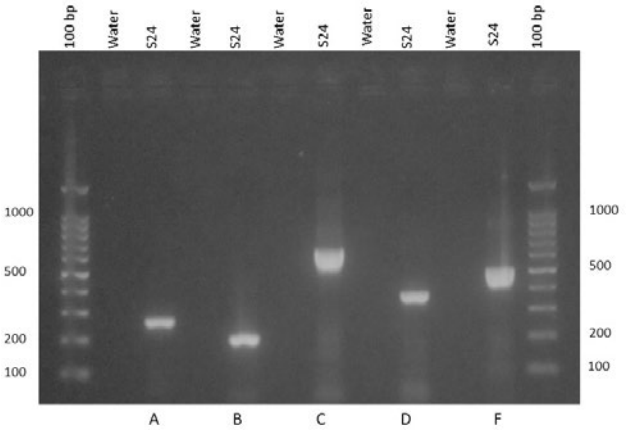

Figure 5. PCR amplification products of antibiotic biosynthetic genes in *Bacillus amyloliquefaciens* (S24), with primers (A) fengycin (FENDF/R) 269 bp; (B) surfactin (SRFAF/R) 201 bp; (C) iturin A (ITUD1F/R) 647 bp; (D) bacillomycin (BMYBF/R) 370 bp; and (E) bacilysin (BCAF/R) 498 bp

Modes of action of antagonistic bacteria

Production of antibiotic genes

The five lipopeptides genes Fengycin, Surfactin, Iturin A, Bacillomycin and Bacilysin were detected in all the *Bacillus* strains (*B. pumilus*S19 and S24, *B. amyloliquefaciens,* and *B. siamensis* (S54). However, the Bacillomycin gene was not detected in the *P. polymyxa* strain (S31) (Table 2; Figure 5).

Cellulase production

The *P. veronii* strain (S40) was the most efficient for production of cellulase (2.64 U mL^{-1}), followed by the three strains of *Bacillus* with S19 producing 1.62 U mL^{-1}, S24 producing 2.13 U $mL^{-1}$and S54 producing 2.51 U mL^{-1}. Cellulase production from *Paenibacillus polymyxa* strain S31 was 1.21 UmL^{-1} (Table 2).

Siderophore production

All the strains produced siderophores. Strain S24 produced the smallest halo (+) from 1 to 10 mm, and the strains S31 and S54 produced the largest halo zones (+++) of 20 to 30 mm (Table 2).

Table 2. Modes of action of selected antagonistic bacteria in dual culture assays with *Phytophthora citrophthora.*

Strain	Hydrolytic enzyme[a]					PGPB traits[b]			Antibiotic molecular screening[a]				
	Lip	Amy	Ure	Prot	Cell (UmL^{-1}) [c]	Sid	Phos	Aux	Fen	Bcin	Bsin	Surf	Itu A
S19	-	+	-	-	1.62±0.12ab	+	++	-	+	+	+	+	+
S24	+	+	-	+	2.13±0.05ab	+	++	-	+	+	+	+	+
S31	-	+	-	+	1.21±0.13a	+++	+	+	+	-	+	+	+
S40	-	+	-	-	2.64±0.06b	+	+++	-	-	-	-	-	-
S54	+	+	-	+	2.51±0.02ab	+++	++	-	+	+	+	+	+

[a] (+) Positive reaction (-) and negative reactions for: Lip , Lipase; Amy, Amylase; Ure, Urease; Prot, protease; Cell, cellulase; Sid, Siderophore production; Phos, phosphate solubilization; Aux, Auxin production; Fn, Fengycin; Bcin, Bacillomycin; Bsin, Bacilysin; Surf,: Surfactin; Itu A, Iturin A.

[b] PGPB: Plant growth-promoting bacteria.+, Growth ranging from 1 to 10 mm; ++, growth ranging from 10 to 20 mm; +++, growth ranging from 20 to 30 mm.

[c]Mean cellulase activity (U mL^{-1}) followed by different letters are significantly different ($P < 0.05$, n = 6; Tukey tests) (± standard errors).

Table 3. *In planta* antagonistic activity of four bioactive bacteria toward *Phytophthora citrophthora.*

Treatments with bioactive bacteria[a]	Mean lesion length(cm)[b]	Average reduction in lesion size (%)[c]
Control	4.9±0.47a[d]	
S24	3.2±0.42bc	35
S31	2.9±0.19b	41
L80	2.4±0.20b	51
M3-16	3.1±0.22c	37

[a] Treatments by bioactive bacteria applied at 10^8 UFC mL^{-1}.
[b] Mean lesion length 10 d after inoculation with *P. citrophthora*. Mean of 40 inoculation points.
[c] Average reduction in lesion size [% reduction = 100 – (Av. lesion size treatment × 100)/Av. lesion size control] was calculated for the treatments that displayed statistically significant differences from the non treated controls.
[d] Means followed by the same letter are not significantly different ($P < 0.05$, n = 40) according to Dunn tests.

Phosphate solubilization

The three *Bacillus* strains produced clear phosphate solubilized zones, ranging from 15 to 20 mm after 9 d. The *Ps. veronii* strain S40 produced the largest zone of more than 20 mm. *Paenibacillus polymyxa* (S31) was the weakest strain for this character, with zones less than 15 mm (Table 2).

Auxin production

Only the strain *Paenibacillus polymyxa* S31 produced IAA (18.5 µg mL^{-1},Table 2).

Suppression of branch canker on clementine mandarin plants

The endophytic and halophilic bacterium strains all reduced ($P<0.0001$; Kuskalwallis tests) the size of necrotic lesions caused by *P. citrophthora* on clementine plants 10 dafter inoculations, compared to the non-treated plants (Figure 4). The strain L80 of *Halomonas elongata* was the most effective for lesion reduction, giving 51% reduction of mean lesion size (Table 3). The other bacteria reduced lesion sizes, proportional reductions not exceeding 41%.

DISCUSSION

The Tunisian citrus industry has flourished in recent years, and this has been partly due to increased clementine production. However, trunk and branch canker caused by *P. citrophthora* represents a serious threat to productive clementine cultivation. Biological control of this new disease has not been previously investigated. In order to establish an eco-friendly strategy for management of *Phytophthor*a, 69 isolates of bacteria from citrus roots were identified and screened for antagonistic activity against *P. citrophthora*. Five strains had strong *in vitro* antagonistic effects against *P. citrophthora*. Molecular characterization allowed us to identify these strains, three of which were *Bacillus*, one was *Paenibacillus*, and the fifth was *Pseudomonas*. Köberl *et al.* (2011) found that *Bacillus* and *Paenibacillus* represent 96% of the antagonists towards phytopathogens in agriculture. Size of the inhibition zone in dual culture was the first criterion used to select the antagonist candidates. The use of PDA as a rich nutrient medium could exclude competition as a mode of action. No physical contact between the isolates and the pathogen in the dual culture tests could be related to the production of antifungal metabolites that inhibit mycelium growth (Montealegre *et al.*, 2003; Lee *et al.*, 2008). The growth inhibition proportion (GI%) of the selected bacteria varied from 74% for *B. pumilus* S19 to 62% for *Ps. veronii* S40. Among those most antagonistic bacteria, *B. amyloliquefaciens* and *P. polymyxa* were selected using a preliminary *in vivo* trial (results not shown) to study their ability to suppress branch canker under greenhouse conditions. In addition, two halophilic bacteria isolated from Tunisian sebkhas were selected. These were *Bacillus pumilus* M3-16 and *Halomonas elongata* L80. These halophilic bacteria produced extracellular antifungal enzymes such as chitinase, glucanase, and protease, and were characterized by tolerance and stability in the presence of extreme conditions (pH, temperature and salts) compared to other antifungal enzymes reported in the literature (Essghaier *et al.*, 2009b, 2010, 2012). Other reports have shown that halophilic bacteria with inability to produce inhibition zones in solid medium co-culture tests, have also demonstrated promising disease inhibition in *in vivo* tests. *Bacillus thuringiensis* strains unable to form inhibition zones in dual cultures were very effective *in vivo* on potato tubers (Sadfi *et al.*, 2001). *Bacillus subtilis* effectively inhibited grey mold on wounded tomatoes but was ineffective *in vitro* on PDA medium (Sadfi-Zouaoui *et al.*, 2008b).

The endophytic bacteria isolated from citrus roots demonstrated abilities to produce cell wall-degrading enzymes such as endoglucanase and protease, which are important for breaking through plant cell walls. Reinhold-Hurek*et al.* (2006) confirmed that endoglucanases are essential for *Azoarcus* sp. to colonize rice roots. Many other studies have confirmed that bacterial endophytes

are mainly recruited from soil via rhizosphere and root systems, to reach xylem and phloem vessels and colonize plants intra- and extra-cellularly (Liu *et al*., 2017). Little is known about endophyte colonization of citrus tissues. Lacava *et al*.(2007) studied the colonization of *Citrus sinensis* by endophytic bacteria, choosing the model of a *K. pneumoniae* strain labelled with GFP genes. They concluded that the endophytic bacterium strain colonized xylem vessels of *C. roseus* branches and roots.

The greenhouse trial of the present study revealed that the endophytic *B. amyloliquefaciens* and the halophilic *B. pumilus* produced similar results, reducing necroses in citrus plants by, respectively, 35 and 37%. Several other studies have demonstrated the ability of *B. amyloliquefaciens* to control *in planta* infections by *Phytophthora* spp. (Chung *et al*., 2005; Anandhakumar *et al*., 2008; Li *et al*., 2014; Zhang *et al*., 2016). Anandhakumar *et al*. (2008) showed that *B. amyloliquefaciens* reduced red core and crown rot diseases of strawberry in a greenhouse trial, exhibiting a similar level of disease control (up to 59%) as the chemical fungicide Aliette®. The second most effective bacterium in the present study was *P. polymyxa,* which reduced necrosis by 41%. Recent publications have also indicated the potential of *P. polymyxa* as a bio-pesticide (Grady *et al*., 2016; Weselowski*et al*., 2016; Luo *et al*., 2018). However, it is the ability of this bacterium to inhibit *Phytophthora* diseases of citrus has not been previously explored.

The greatest *in vitro* inhibition of *P. citrophthora* was obtained with *H. elongata*, with 51% reduction of host necrosis. This is the first record of potential for *H. elongata*as a biocontrol agent against *Phytophthora* sp. In order to study the PGPB traits of this bacterium, direct and indirect mechanisms to promote citrus growth were evaluated. Direct mechanisms include increased availability of plant nutrients (biofertilization), from phosphate solubilization and azote fixation. According to the biochemical test 'Gallerie API 20 NE', all the five strains were able to reduce atmospheric azote (N_2) to nitrite (NO_2^-) and then to nitrate (NO_3^-). In addition, the *P. polymyxa* S31 strain could synthesize IAA enhances plant cell growth and proliferation (Grady *et al*., 2016). The indirect mechanism was demonstrated by the abilities of all the candidate bioactive bacteria to produce siderophores, lytic enzymes such as lipase, protease and cellulase, and to possess most of the lipopeptide genes. Interactions between lipopeptides can become synergistic to enhance their respective activities (Romero *et al*., 2007; Malfanova *et al*., 2012; Li *et al*., 2014). Previous studies have highlighted the role of *Bacillus* lipopeptides in biological control of plant diseases (Ongena and Jaques, 2008; Mora *et al*., 2015; Paraszkiewicz *et al*., 2017). Beside their involvement in antifungal and antimicrobial activities, some bacteria also facilitate root colonization and modulate plant immunity (Ongena and Jaques, 2008).

CONCLUSIONS

This study has demonstrated potential to overcome the new serious disease in clementine orchards by adopting an ecological biocontrol approach. After screening endophytic bacteria from citrus roots, five bacteria displayed strong antagonistic traits as *in vitro* pathogen inhibition rates, metabolization of different carbon sources, PGPB traits, and antibiotic production. In the greenhouse trial, the strains L80 of *H. elongata* and S31 of *P. polymyxa* showed the greatest potential as candidates for control the disease. This is the first biological control study of clementine branch canker. The bacterial strains S31 and L80 offer a good basis for developing efficient biofungicides, and should be further investigated under field conditions.

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