Microbiological Research 206 (2018) 50–59

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

A novel *Burkholderia ambifaria* strain able to degrade the mycotoxin fusaric acid and to inhibit *Fusarium* spp. growth

Ester Simonetti, Irma N. Roberts*, Marcela S. Montecchia, Flavio H. Gutierrez-Boem, Federico M. Gomez, Jimena A. Ruiz*

Instituto de Investigaciones en Biociencias Agrícolas y Ambientales, Universidad de Buenos Aires, CONICET, FAUBA. Av. San Martín 4453, C1417DSE, Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Barley
Burkholderia ambifaria
Fusaric acid
Fusarium

ABSTRACT

Fusaric acid (FA) is a fungal metabolite produced by several *Fusarium* species responsible for wilts and root rot diseases of a great variety of plants. *Bacillus* spp. and *Pseudomonas* spp. have been considered as promising biocontrol agents against phytopathogenic *Fusarium* spp., however it has been demonstrated that FA negatively affects growth and production of some antibiotics in these bacteria. Thus, the capability to degrade FA would be a desirable characteristic in bacterial biocontrol agents of *Fusarium* wilt. Taking this into account, bacteria isolated from the rhizosphere of barley were screened for their ability to use FA as sole carbon and energy source. One strain that fulfilled this requirement was identified according to sequence analysis of 16S rRNA, *gyrB* and *recA* genes as *Burkholderia ambifaria*. This strain, designated T16, was able to grow with FA as sole carbon, nitrogen and energy source and also showed the ability to detoxify FA in barley seedlings. This bacterium also exhibited higher growth rate, higher cell densities, longer survival, higher levels of indole-3-acetic acid (IAA) production, enhanced biofilm formation and increased resistance to different antibiotics when cultivated in Luria Bertani medium at pH 5.3 compared to pH 7.3. Furthermore, *B. ambifaria* T16 showed distinctive plant growth-promoting features, such as siderophore production, phosphate-solubilization, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, *in vitro* antagonism against *Fusarium* spp. and improvement of grain yield when inoculated to barley plants grown under greenhouse conditions. This strain might serve as a new source of metabolites or genes for the development of novel FA-detoxification systems.

1. Introduction

Fusarium species are ubiquitous soil inhabiting fungi, including phytopathogenic and non-phytopathogenic strains. Fusaric acid (FA, 5-butylpyridine-2-carboxylic acid) is a toxin produced by several *Fusarium* spp., which was shown to contribute to wilt and rot diseases of a wide variety of crops (Pegg, 1981). This compound negatively affects plant cells viability by different mechanisms, including reduction in the availability of metals (Chakrabarti and Ghosal, 1989), enhancement of ROS (reactive oxygen species) levels (Singh and Upadhyay, 2014) and alteration of membrane permeability and potential (Marrè et al., 1993; Pavlovkin et al., 2004).

Production of FA by different phytopathogenic *Fusarium* spp. has been evaluated *in vitro* and *in planta*. In most cases, a positive correlation between FA production and virulence was observed (Pegg, 1981; El-Hassan et al., 2007). Moreover, FA has been found at high levels in tissues, fruits, seedlings and cereal grains of plants infected by FA producers (Pegg, 1981; Bacon and Hinton, 1996; Li et al., 2013) and a synergistic interaction between FA and other mycotoxins, such as fumonisins and deoxynivalenol has been reported (Smith, 1992; Bacon et al., 1995). These results highlight the importance of FA contamination for human and animal health.

Soil-borne diseases caused by *Fusarium* spp. are difficult to control using fungicides and once a field is infected, these fungi can survive in soil and plant debris for long periods. A promising strategy for the suppression of these soil-borne pathogens is by the use of bacterial biocontrol agents. Strains belonging to the genera *Bacillus* and *Pseudomonas* have been shown to contribute to *Fusarium* wilt-

Abbreviations: FA, fusaric acid (5-butylpyridine-2-carboxylic acid); ACC, 1-aminocyclopropane-1-carboxylate; ROS, reactive oxygen species; MT, metal trace; PHAs, polyhydroxyalkanoates; NA, nutrient agar; M9wN, modified M9 medium without NH_4Cl; PKV, Pikovskaya agar medium; SI, phosphate solubilization index; IAA, indole-3-acetic acid; CAS, chrome azurolsulphonate; CFS, cell-free supernatant; μ, specific growth rate; MIC, minimal inhibitory concentration

* Corresponding authors at: Cátedra de Microbiología Agrícola. Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, C1417DSE. Ciudad Autónoma de Buenos Aires, Argentina.

E-mail addresses: iroberts@agro.uba.ar (I.N. Roberts), jruiz@agro.uba.ar (J.A. Ruiz).

http://dx.doi.org/10.1016/j.micres.2017.09.008
Received 1 March 2017; Received in revised form 7 September 2017; Accepted 20 September 2017
Available online 22 September 2017
0944-5013/

suppressiveness (Weller et al., 2002) and have been used to control diseases caused by these fungi (Chin-A-Woeng et al., 1998; Bacon et al., 2001; Duffy et al., 2004; Saikia et al., 2009). However, it has been demonstrated that the presence of FA is highly inhibitory for many *Bacillus* species (Bacon et al., 2006) and negatively affects growth of *P. protegens* by sequestering iron (Ruiz et al., 2015) and increasing ROS levels (Bernar and Ruiz, 2016). Moreover, detrimental effects of FA on the production of some antifungal compounds, such as 2,4-diacetylphloroglucinol and phenazine-1-carboxamide, have been reported in fluorescent *Pseudomonas* spp. (Schnider-Keel et al., 2000; Notz et al., 2002; Duffy et al., 2004; van Rij et al., 2004; van Rij et al., 2005; Quecine et al., 2015). Thus, the production of FA by *Fusarium* spp. is unfavorable for the biocontrol activity of these bacteria, negatively affecting their competition against soil-borne pathogens. Taking this into account, the purpose of the present work was to isolate and characterize FA-degrading bacteria, which might be useful in developing novel strategies for the biocontrol of FA-producing phytopathogenic *Fusarium* spp. Bacteria collected from the rhizosphere of barley plants were screened in minimal medium supplemented with FA to select those which could use this toxin as sole C and energy source. An isolate able to grow under this condition was further tested for its ability to degrade FA *in vitro* and *in planta*. In addition to its phylogenetic and biochemical characterization, antifungal activity and several plant growth-promoting traits were determined *in vitro* and under greenhouse conditions. Moreover, since the strain was isolated from barley plants growing in acidic soil, we also investigated the effect of pH on bacterial growth, antibiotic resistance, biofilm formation, long-term viability, antifungal activity and indole-3-acetic acid (IAA) production.

2. Materials and methods

2.1. Sample collection and bacterial isolation

Six healthy barley plants (*Hordeum vulgare* L. cv. Scarlett) along with their rhizosphere soil were collected at 168 days after sowing (DAS) from a field in Junín (latitude 34°35′S; longitude 60°56′W), Buenos Aires Province, Argentina, placed into a sterile plastic bag, and stored at 4 °C until the next day. Although no previous history of fungal diseases caused by *Fusarium* spp. has been recorded in this field where the sampling was carried out, economically important crop diseases caused by *Fusarium* spp. are known in the Flooding Pampa, and are difficult to control, even with chemical substances. Rhizosphere suspensions were obtained by placing 13 g of roots and the soil associated to them in 100 ml of sterile PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4, pH 7.4). The suspension of soil and roots was sonicated during 10 min in an ultrasonic bath (Type XB2, Grant Instruments, Cambridge, England) and shaken at 150 rev min^{-1} during 30 min. Afterwards, ten-fold serial dilutions from the rhizosphere suspension were plated in Lysogeny Broth (LB), S1 (Gould et al., 1985) and Trypan Blue Tetracycline (TBT) (Hagedorn et al., 1987) agar and incubated at 30 °C. Resulting colonies were streaked in the same medium and further purified by streaking them in LB. Finally, single colonies were streaked on M9 minimal medium agar plates (Miller, 1972) supplemented with 1 mM $MgSO_4$, 0.1% (v/v) metal trace (MT) solution (Lageveen et al., 1988) and 2.5 mM FA as sole C source, prepared as earlier described (Ruiz et al., 2015).

2.2. Isolate identification by PCR amplification and DNA sequencing

The nearly complete sequence of the 16S rRNA gene and the partial *gyrB* and *recA* genes sequences were obtained as previously described (Weisburg et al., 1991; Spilker et al., 2009). Amplification products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Germany) and sequenced on both strands at Macrogen (Seoul, Korea). The obtained sequences were compared with those from GenBank using BLASTN 2.2.16 (Altschul et al., 1990). A phylogenetic tree using *gyrB* sequences was constructed using the Neighbor-Joining method with the Kimura 2-parameter model. Bootstrap analysis with 1000 replicates was performed to assess the support of the clusters. MEGA version 6 (Tamura et al., 2013) software was used for all analyses.

2.3. Biochemical characterization

The API20NE identification system (bioMérieux, France) was used to test nitrate reduction, gelatin and aesculin hydrolysis, arginine dihydrolase and cytochrome *c* oxidase activity, as well as assimilation of different C sources among other biochemical tests. The API20E identification system was used to analyze lysine and ornithine decarboxylase activity and tryptophan deaminase activity. Production of polyhydroxyalkanoates (PHAs) was tested by growing bacterial cells in 0.5 Nitrogen E_2 (0.5 NE_2) medium (Huisman et al., 1992) supplemented with 1% (w/v) glucose, 1 mM $MgSO_4$, and 0.1% (v/v) MT solution during 24 h at 30 °C and staining the cells with Nile Blue (Ostle and Holt, 1982). Hemolytic activity was checked in nutrient agar (NA) plates containing 10% (v/v) sterile and defibrinated sheep blood.

2.4. Growth curves with FA as sole C and/or N source

To evaluate the growth of bacteria with FA as sole C and energy source, cells from an overnight culture grown in M9 minimal medium supplemented with 0.6% (w/v) glucose, 1 mM FA, 1 mM $MgSO_4$ and 0.1% (v/v) MT solution, were inoculated into fresh M9 medium supplemented with 2.5 mM FA as sole C source, to give an initial OD_{600} of 0.05. Cultures were grown in Erlenmeyer flasks at 180 rev min^{-1} and 30 °C and samples were withdrawn at different times to estimate bacterial growth by reading the OD_{600} and to measure the FA concentration in the supernatant as earlier described (Ruiz et al., 2015).

To analyze growth with FA as sole N source, bacteria were precultured in M9 glucose-FA medium as described above and afterwards inoculated into a modified M9 medium, without NH_4Cl (M9wN), supplemented with 0.6% (w/v) glucose, 1 mM FA, 1 mM $MgSO_4$ and 0.1% (v/v) MT solution. These cultures were used to inoculate Erlenmeyer flasks containing M9wN supplemented with 0.6% (w/v) glucose as C source, and 2.5 mM FA as C and sole N source. All cultures were started with an initial OD_{600} of 0.05 and incubated as mentioned above for determination of the OD_{600}.

The utilization of FA as sole C and N source was evaluated in M9wN supplemented with 2.5 mM FA, similar to that described in the previous paragraph. Three independent cultures were used for each condition.

2.5. FA detoxification in barley seedlings

Barley seeds were surface-desinfected with 3% NaClO and placed in a humid chamber for 24 h to allow germination. Germinated seeds were incubated during 2 h at 30 °C with bacterial suspensions containing 10^9 CFU ml^{-1} in PBS buffer. Bacterial suspensions were prepared as follows: bacteria were grown in M9 supplemented with 2.5 mM FA as sole C source,1 mM $MgSO_4$ and 0.1% (v/v) MT solution during 24 h at 30 °C, centrifuged at 6150*g* to obtain the pellet, washed twice with PBS buffer and finally resuspended in PBS. Germinated seeds incubated in PBS buffer were used as controls. After incubation, the seeds were placed on the surface of sterile Whatman N°1 paper, moisturized with sterile water in Petri dishes. Another piece of paper was placed covering the seeds. A three ml volume of 2.5 mM or 5 mM FA solutions was added to the paper placed above the seeds. Petri dishes with the addition of three ml sterile water were prepared as controls. Plates were incubated in a humid chamber in the dark. After 72 h of incubation, the total length of the root system (sum of the length of every root) and the coleoptile length were measured on each seedling. A total of 20 seedlings were evaluated for each treatment.

2.6. Effect of pH on growth, long-term viability, antibiotic resistance and biofilm formation

B. ambifaria T16 was isolated from the rhizosphere of barley plants collected from an agricultural field where soil pH was 5.6 (Magliano et al., 2014). Taking this into account, we decided to evaluate the effect of acidic and neutral pH (pH 5.3 and 7.3) on different characteristics of *B. ambifaria* T16. Cells were grown in LB broth containing 100 mM Mes buffer at pH 5.3 or 100 mM Mops buffer at pH 7.3. Growth curves in LB at pH 5.3 and 7.3 were started with an initial OD_{600} = 0.05. Liquid cultures were grown in Erlenmeyer flasks at 180 rev min^{-1} and 30 °C. Samples were withdrawn at different times to estimate bacterial growth by reading the OD_{600} and to determine the CFU ml^{-1} on LB agar plates. Three independent cultures were used for each pH condition.

In order to test long-term viability of bacterial cells growing at different pHs, at least 15 different colonies grown in LB agar plates adjusted to pH 5.3 or 7.3 were transferred weekly to new plates containing the same medium for a period of 102 days.

Resistance of the bacterial cells grown at different pHs to the antibiotics potassium tellurite, cefotaxime, ciprofloxacin and sulfamethoxazole-trimethoprim was assessed in LB medium adjusted to pH 5.3 or 7.3 by determination of the minimal inhibitory concentration (MIC). Cultures were inoculated with an initial OD_{600} = 0.05 and grown overnight with shaking in 96-well microtiter plates at 30 °C. Two-fold serial dilutions of the respective antibiotic were performed in a volume of 150 µl of LB medium buffered at pH 5.3 or 7.3. A growth control without antibiotic was also included in each case. The MIC was determined as the concentration of the antibiotic that completely inhibited growth. The experiment was performed in triplicate.

Biofilm formation was evaluated in polystyrene 96 well-plates containing 150 µl of LB adjusted to pH 5.3 or 7.3 supplemented with 25 mM glucose. The bacterial inocula were grown overnight in LB and an aliquot of these cultures were inoculated into the wells with an initial OD_{600} = 0.05. Plates were incubated during 48 h at 30 °C in a humid chamber and the proportion of attached bacteria was evaluated as earlier described (Merritt et al., 2005) by crystal violet staining and subsequent resuspension of the dye in dimethyl sulfoxide (DMSO). The experiment was performed three times with three independent cultures each time and 10 replicates of each culture.

2.7. In vitro characterization of plant growth-promoting related traits of the strain T16

The ability of bacteria to utilize 1-aminocyclopropane-1-carboxylate (ACC) as sole N source was tested in DF (Dworkin and Foster, 1958) minimal medium as previously described by Penrose and Glick, (2003). Bacterial culture with an initial OD_{600} = 0.05 was used to inoculate DF minimal medium supplemented with 2 g l^{-1} $(NH_4)_2SO_4$, or with 3 mM ACC as N source. *P. brassicacearum* DSM 13227 and *P. fluorescens* DSM 8568 (Long et al., 2010) were used as positive controls, while *P. protegens* Pf-5 (Howell and Stipanovic, 1979) was used as a negative control.

The ability of bacteria to solubilize phosphate was assessed using Pikovskaya medium (PKV) medium (Pikovskaya, 1948) amended with 5 g l^{-1} $(Ca)_3(PO_4)_2$. Bacteria were spot-inoculated onto the center of PKV plates and incubated at 30 °C during 8 days. The diameters of the clearing zones (halos) surrounding the colonies were measured. The phosphate solubilization index (SI) was calculated as the ratio of the total diameter (colony + halo) to the colony diameter (Edi Premono et al., 1996). *P. protegens* Pf-5 was used as a positive control. The experiment was done in triplicate.

IAA production was quantified spectrophotometrically at 530 nm according to the method described by Glickman and Dessaux (1995). Cultures were grown in LB broth buffered at pH 5.3 or pH 7.3, as described above, supplemented with 1 mg ml^{-1} tryptophan and incubated for 48 and 72 h at 30 °C and 180 rev min^{-1}. After incubation, bacterial cultures were diluted to a similar OD (OD_{600} = 9) with LB broth buffered at pH 5.3 or 7.3, as appropriate. Bacterial suspensions were pelleted by centrifugation at 12,500*g* for 15 min and the supernatants were used for IAA quantification using IAA (Sigma-Aldrich, USA) as standard. The cultures were set up in triplicate and the experiment was repeated twice.

Siderophore production was tested qualitatively by the O-CAS assay (Pérez-Miranda et al., 2007). Briefly, bacteria were streaked on LB agar plates and incubated at 30 °C for 24 h. An overlay containing CAS medium with agarose as gelling agent was applied over the colonies and the plates were further incubated for 48 h at 30 °C. The presence of a yellowish-orange halo around the colonies was considered indicative of siderophore production.

2.8. Effect of bacterial inoculation on barley plants grown under greenhouse conditions

For inoculation with *B. ambifaria* T16, barley seeds were surface-disinfected and incubated as described above with a bacterial suspension containing 10^7 CFU ml^{-1}. This suspension was prepared by culturing bacteria in LB broth during 18 h at 30 °C, centrifuging at 6150 g, washing the cells in PBS buffer and finally adjusting the suspension to an OD_{600} = 0.2. Inoculated and control seeds (incubated with PBS buffer) were placed on filter papers soaked with sterile water in Petri dishes and incubated at room temperature in an humid chamber in the dark during 72 h. Germinated barley seedlings were transferred into six liter pots (six plants per pot) with a mixture of soil: perlite: vermiculite (1: 1: 1), sterilized by tyndallization (100 °C, 1 h during three consecutive days). The chemical characteristics of the substrate mixture used were pH (H_2O) 7.8, organic matter 9.38%, N-NO_3 126.3 mg $kg^{-1}$and extractable P 133.0 mg kg^{-1} (Bray-1). The plants were irrigated with tap water to keep substrate at field capacity. The experiment was carried out under greenhouse conditions with natural light and temperature. The assay consisted of two treatments, plants inoculated and non-inoculated with *B. ambifaria* T16. The pots were arranged in four completely randomized blocks. Aerial vegetative tissues and grains were sampled at physiological maturity (185 DAS). At 30 DAS, bacterial root colonisation was evaluated as follows: roots from three plants per treatment were submerged in PBS buffer, sonicated during 10 min in an ultrasonic bath (Type XB2, Grant Instruments, Cambridge, England), shaken at 150 rev min^{-1} during 30 min, serially diluted and plated on TBT to obtain CFU [g of fresh weight of roots] $^{-1}$.

Aerial vegetative tissues harvested at physiological maturity, were oven-dried to determine dry weight. The dried grains were milled and analyzed for total N content after Kjeldahl digestion (Baethgen and Alley, 1989). Spike dry weight, spike number, grain yield per pot and grain fraction > 2.5 mm were evaluated.

2.9. Antifungal activity

The bacterial isolate was tested for its ability to inhibit mycelial growth of *F. graminearum, F. oxysporum* and *F. solani* by dual plate assays. *F. oxysporum and F. graminearum* strains were isolated from soil cultivated with wheat in Tres Arroyos, Buenos Aires Province, Argentina (Silvestro et al., 2013) and *F. solani* was isolated from barleýs roots in Junín, Buenos Aires Province, Argentina. Fungi were routinely cultivated on Potato Dextrose Agar (PDA, Merck) at 25 °C. Six mm diameter mycelial plugs were taken from the edge of a growing fungal colony and spot-inoculated onto the center of a Petri dish containing PDA and NA (1:1 v/v). Bacteria were streaked in a straight line at 2.5 cm from the fungal colony and the plate was incubated at 25 °C (Plate B). Plates containing *Fusarium* strains in the absence of bacteria were also incubated at 25 °C (Plates A). The diameter of the fungal colony in absence (A) or presence (B) of bacteria was recorded and the percent inhibition of mycelial growth was calculated by using the following formula: (A-B)/(Ax100). The assay was repeated twice with

three different bacterial colonies each time.

Antifungal activity of bacterial cell-free supernatants (CFSs) was analyzed as follows: overnight cultures grown in unbuffered LB broth or LB broth buffered at pH 5.3 or 7.3 were adjusted to an OD_{600} = 9 and centrifuged during 15 min at 12,500*g* at 4 °C. The supernatants were filter-sterilized (0.22 μm) and mixed with 2X PDA medium at 45 °C, to give a final concentration of CFS of 10, 25 or 50% (v/v) (Plates B). *Fusarium* spp. strains were inoculated in the center of the plates as described above. Control plates containing LB broth instead of CFS were also performed (Plates A). All experiments were performed twice with three independent cultures each time. The inhibition of the mycelial growth was calculated as described for the dual plate assays.

2.10. Statistical analysis

Data were subjected to an analysis of variance (ANOVA) followed by Fisher LSD multiple-range test, using Sigma Plot software v. 11 (Systat software, Inc.). P-values were considered significant when they were lower than 0.05.

3. Results

3.1. Isolation and identification of a FA-degrading bacterium

About 300 hundred bacterial colonies recovered from the rhizosphere of barley plants were tested for their ability to use FA as sole C and energy source. One bacterial strain, designated T16, originally isolated in TBT medium, fulfilled this characteristic. Interestingly, this bacterium was present in high numbers, around 1×10^5 CFU g^{-1} roots in the samples analyzed.

The 16S rRNA, *gyrB* and *recA* genes sequences determined for strain T16 are publicly available through the GenBank accession numbers KX702306, KX702307 and KX702308, respectively. Pairwise comparison of 16S rRNA gene sequence of the T16 isolate, as determined with the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon/identify), revealed that it belongs to *Burkholderia cepacia* complex (Bcc) species with the highest similarity value (99.78%) with the type strain of *Burkholderia ambifaria* (AMMD). Sequence analysis of *gyrB* and *recA* genes (Payne et al., 2005; Tabacchioni et al., 2008), both used for species-level discrimination of Bcc species, confirmed the identification of the T16 strain as *B. ambifaria*. Phylogenetic analysis based on partial *gyrB* gene sequences is shown in Fig. 1. This strain was deposited in the collection Banco Nacional de Microorganismos, Argentina (WDCM 938) under number BNM 561.

3.2. Biochemical characterization of B. ambifaria T16

B. ambifaria T16 showed the following phenotypic and biochemical characteristics in coincidence with other bacterial strains belonging to the same species (Coenye et al., 2001; Vanlaere et al., 2008): Gram negative straight rods; able to grow at 30 °C and 37 °C, but not at 42 °C; positive for cytochrome *c* oxidase, β-galactosidase, gelatinase, lysine decarboxylase, aesculin hydrolase and able to reduce nitrate to nitrite; negative for ornithine decarboxylase, tryptophanase, arginine hydrolase and urease; able to assimilate glucose, L-arabinose, D-mannose, N-acetylglucosamine, gluconate, caprate, adipate, L-malate, citrate and phenylacetate and negative for maltose assimilation. Absence of hemolytic activity. In addition, *B. ambifaria* T16 was able to accumulate PHAs granules when grown under conditions of N limitation and C (glucose) excess.

3.3. Growth curves of B. ambifaria T16 with FA as sole C and/or N source

We first evaluated the growth of *B. ambifaria* T16 in M9 minimal medium supplemented with 2.5 mM FA as sole C and energy source (Fig. 2). In overday cultures, a long lag phase of about 2.5 h was observed, as well as a very slow growth rate (μ) during the first 12 h of cultivation, reaching an $OD_{600} \sim 0.15$. Finally, growth was evaluated after overnight cultivation. As it can be observed in the graph, the OD_{600} of the cultures duplicated after 13 h of growth and from that time point, the growth rate increased markedly ($\mu = 0.243\ h^{-1}$). Cells reached the stationary phase of growth after 20 h of incubation at 30 °C. Analysis of the FA content in the supernatant of the cultures demonstrated that 24% of the initial FA has been degraded after 13 h of growth and 80% after 19.5 h. No remaining FA was detected after 24 h of cultivation.

As FA contains a N atom in the aromatic ring, we tested if *B. ambifaria* T16 could also use this compound as sole N source by growing bacteria in M9wN supplemented with 0.6% glucose and 2.5 mM FA (Fig. 2). Similarly to that observed when FA was used as sole C source, cultures showed a very slow growth rate during the first 12 h of incubation, and after that time, growth rate increased. The growth rate of *B. ambifaria* T16 when growing with FA as sole N source was $0.147\ h^{-1}$, almost half the value obtained with FA as sole C source and N supplied as NH_4Cl. After 24 h of cultivation, the growth rate decreased, but the OD_{600} continued increasing until 37 h, time at which the cultures reached the stationary phase.

Finally, bacterial growth with FA as sole C and N source was evaluated in M9wN supplemented with 2.5 mM FA (Fig. 2). The growth rate obtained under this condition ($\mu = 0.158\ h^{-1}$) was closer to that obtained when FA was used as sole N source. Cultures reached stationary phase after 23 h of growth.

3.4. FA detoxification in barley seedlings

Results described in the previous paragraphs demonstrated that *B. ambifaria* T16 was able to degrade FA *in vitro*, using the mycotoxin as sole C and N source. Next, we decided to evaluate its FA degrading capacity *in planta* and test if the inoculation of barley seeds with *B. ambifaria* T16 could attenuate the toxic effect of the mycotoxin on the growth of barley seedlings. Fig. 3 shows that FA at a concentration of 2.5 and 5 mM negatively affects the growth of 3-day-old barley seedlings, causing a significant reduction in roots (Fig. 3A) and coleoptile (Fig. 3B) lengths. Inoculation of barley seeds with *B. ambifaria* T16 significantly reduced the negative impact of FA on roots (Fig. 3A) and coleoptiles development (Fig. 3B).

3.5. Effect of pH on growth, long-term viability, antibiotic resistance and biofilm formation in B. ambifaria T16

Growth curves of *B. ambifaria* T16 in LB broth buffered at pH 7.3 and 5.3 are shown in Fig. 4A. The specific growth rate of cells cultured in LB at pH 5.3 ($\mu = 0.3632\ h^{-1}$) was slightly higher than the growth rate of cells in LB at pH 7.3 ($\mu = 0.3260\ h^{-1}$). Besides, during late exponential and stationary phase, bacterial cultures growing at pH 5.3 exhibited higher OD_{600}values than the obtained for the cultures growing at pH 7.3. This increase in the OD_{600} was accompanied by an increase in the number of viable cells, with higher CFU counts for the cultures grown at pH 5.3. After 18 h of growth, the CFU count for the cultures grown in LB at pH 5.3 was $1.1 \times 10^{10} \pm 5.6 \times 10^8$ CFU ml^{-1}, while for the cultures grown in LB at pH 7.3 a value of $7.8 \times 10^9 \pm 3.5 \times 10^8$ CFU ml^{-1} was obtained. Microscopic observation of the cells grown at both pH conditions revealed no noticeable differences in their shape or size (data not shown).

Furthermore, we examined long-term viability of *B. ambifaria* T16 growing in LB agar plates at pH 5.3 and pH 7.3. The results obtained showed that 100% of the colonies cultivated at pH 5.3 were still viable after 104 days of incubation, while only 20% of the colonies growing in LB agar pH 7.3 were able to grow after 70 days, and finally none of them were viable after 82 days of incubation.

The tolerance of *B. ambifaria* T16 to different antibiotics was tested in LB medium at pH 7, revealing that this strain was highly resistant to

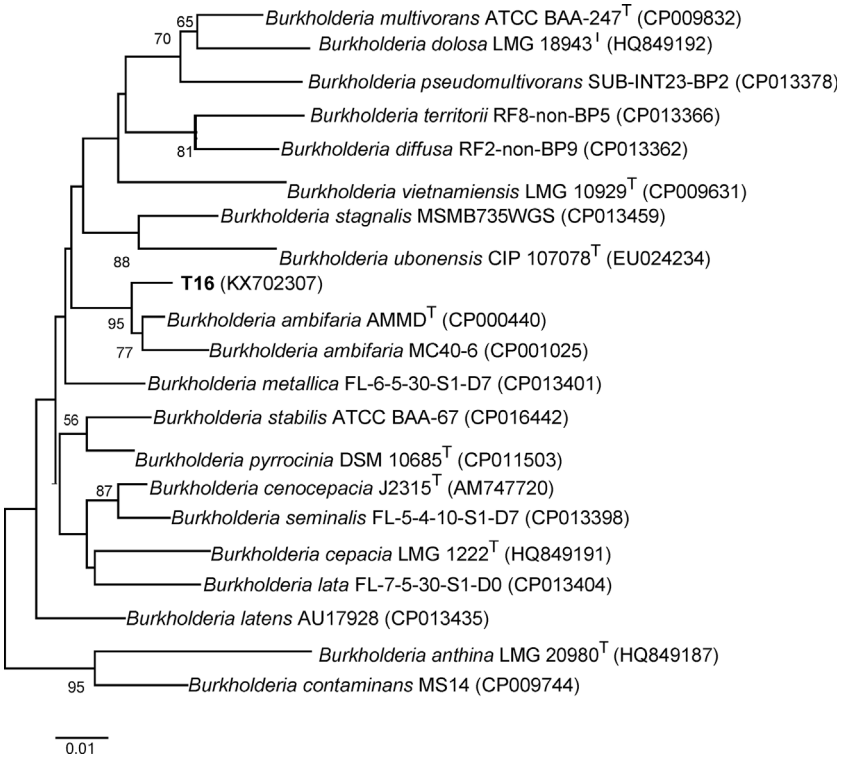

Fig. 1. Phylogenetic tree based on partial *gyrB* gene sequences of T16 strain and established *Burkholderia cepacia* complex species. The consensus tree was constructed using the Neighbor-Joining method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together in the bootstrap test (from 1000 replicates) is shown next to branches if greater than 50%. The scale bar indicates the number of substitutions per site.

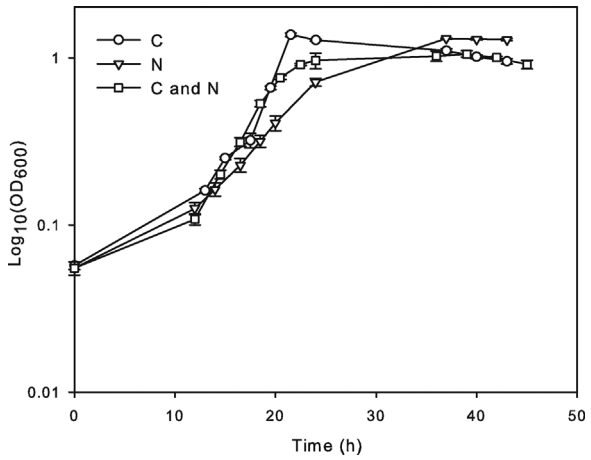

Fig. 2. Growth curves of *B. ambifaria* T16 grown in M9 minimal medium supplemented with 2.5 mM fusaric acid as sole carbon source (C), as sole nitrogen source (N) or as sole C and N source (C and N). OD_{600} data are mean values for three independently grown cultures and error bars represent standard deviations of the mean.

most of the more commonly used antibiotics (ampicillin, tetracycline, streptomycin, chloramphenicol, kanamycin and gentamicin), but showed sensitivity towards potassium tellurite, cefotaxime, ciprofloxacin and sulfamethoxazole-*trimethoprim*. We decided to explore if the tolerance to potassium tellurite, cefotaxime, ciprofloxacin and sulfamethoxazole-trimethoprim increased by culturing cells at acid pH, by comparing the MICs obtained for this bacterium growing in LB at pH 5.3 and pH 7.3. As shown in Table 1, potassium tellurite was the only compound for which no difference was observed between the MIC obtained at neutral and acid LB. On the contrary, for the antibiotics cefotaxime, ciprofloxacin and sulfamethoxazole-trimethoprim, B. ambifaria T16 showed higher MIC values at pH 5.3 than at pH 7.3, indicating a higher tolerance to these compounds at acid pH.

Finally, biofilm formation was evaluated in LB medium buffered at pH 5.3 and 7.3 supplemented with 25 mM glucose. The results obtained (Fig. 4B) clearly showed that the proportion of attached bacteria was significantly higher when they were grown under acidic pH conditions in comparison with neutral pH conditions.

3.6. Plant growth promoting traits of B. ambifaria T16

Several plant growth promoting traits were assayed in *B. ambifaria* T16. As shown in Fig. 4C, *B. ambifaria* T16 released IAA when cultivated in LB medium at acid and neutral pH. The level of production of these phytohormones was significantly higher at pH 5.3 compared to pH 7.3., after 48 and 72 h of incubation at 30 °C.

Bacterial growth on DF minimal medium containing ACC as sole N source is a consequence of ACC deaminase activity. *B. ambifaria* T16 was able to grow under this condition, reaching an $OD_{600} \sim 1.2$ after 24 h of incubation. The two positive control strains *P. brassicacearum* DSM13227 and *P. fluorescens* DSM8568, growing under the same conditions, reached an $OD_{600} = 1.9$ and 1.7, respectively. On the other hand, *P. protegens* Pf-5, which does not harbor the *acdS* gene encoding ACC deaminase (Loper et al., 2012) did not grow on DF-ACC medium.

B. ambifaria T16 also showed phosphate-solubilizing activity in PKV agar, with a SI of 2.4 after 8 days of incubation, similar to that obtained for *P. protegens* Pf-5 (Fig. 5A). Moreover, *B. ambifaria* T16 produced siderophores, as indicated by the presence of yellowish-orange halos around the colonies (Fig. 5B) in the O-CAS assay (Pérez-Miranda et al., 2007).

A pot experiment under greenhouse conditions was carried out in order to test the effect of inoculation with *B. ambifaria* T16 on the growth, grain yield and quality of barley plants. At 30 DAS, colonisation of *B. ambifaria* T16 was evaluated on root surfaces, obtaining 2×10^4 CFU [g of fresh weight of roots]$^{-1}$. When plants reached physiological maturity, spike dry weight, spikes number and grain yield per pot were significantly higher ($P < 0.05$) in plants inoculated with *B. ambifaria* T16 compared to non-inoculated control plants (Table 2). Grain size (fraction > 2.5 mm) was slightly lower in inoculated plants compared to control plants, while grain N content and plant aerial biomass did not show significant differences between treatments (Table 2).

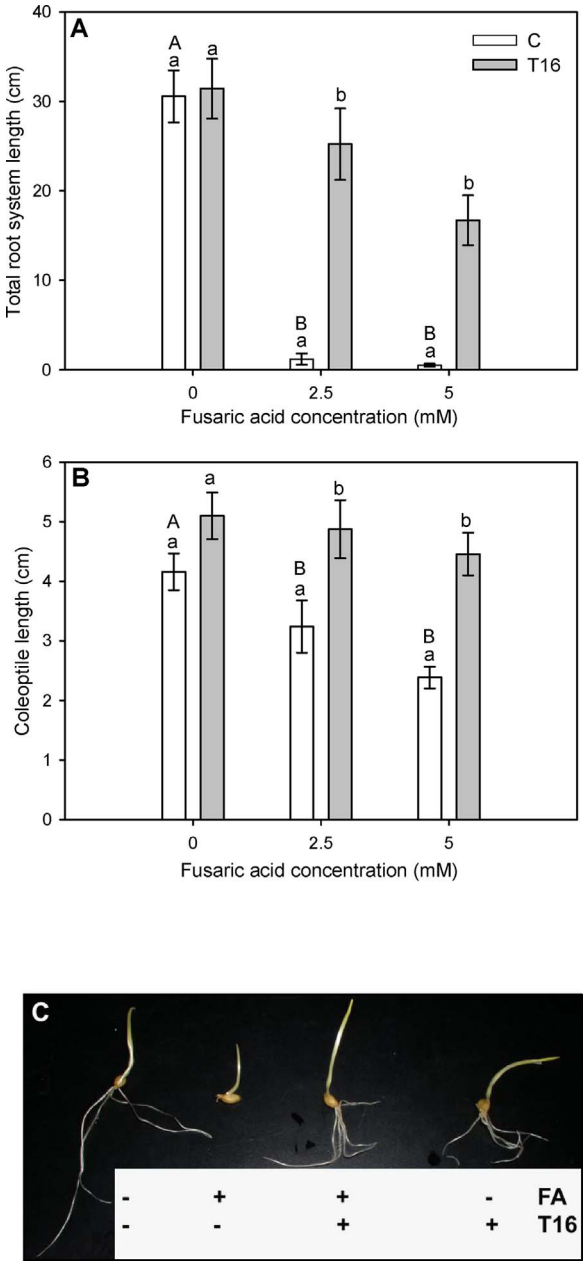

Fig. 3. Fusaric acid (FA) detoxification in barley seedlings by *B. ambifaria* T16. Data represent mean values ± standard deviation (n = 20) of total root length (**A**) and coleoptile length (**B**) of seedlings treated with FA (0, 2.5 or 5 mM), inoculated with 10^9 CFU ml^{-1} *B. ambifaria* T16 (T16) or non- inoculated controls (C). Different lower case letters over the bars indicate significant differences between mean values from inoculated treatments and non-inoculated controls for each FA concentration ($P < 0.001$). Different capital letters over the bars denote a statistically significant difference between mean values from non-inoculated controls subjected to different FA concentrations ($P < 0.001$). (**C**) Photographic image of barley seedlings treated (+) or non-treated (−) with 5 mM FA, and inoculated (+) or non-inoculated (−) with *B. ambifaria* T16.

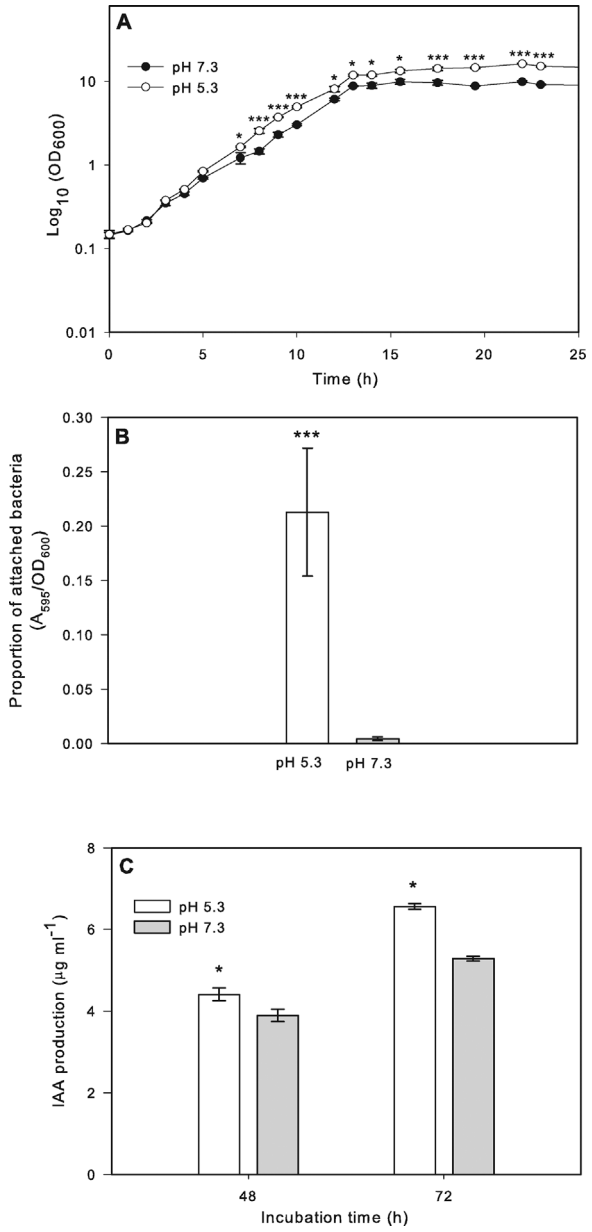

Fig. 4. (**A**) Growth curves of *B. ambifaria* T16 in LB broth buffered at pH 5.3 or pH 7.3. (**B**) Biofilm formation of *B. ambifaria* T16 grown in LB buffered at pH 5.3 or pH 7.3. Biofilm formation was assessed by determining the proportion of attached and non-attached bacteria (A_{595}/OD_{600}). (**C**) IAA production in LB broth buffered at pH 5.3 and pH 7.3. Presented data (**A, B, C**) are mean values from three independent cultures and the error bars indicate standard deviations of the mean. Asterisks denote a statistically significant difference between means values from both pH conditions (*$P < 0.05$; ***$P < 0.001$).

In addition, the antifungal activity of cells and CFS of *B. ambifaria* T16 against several *Fusarium* species was tested (Fig. 6). Dual plate assays demonstrated a significant reduction in the growth of the fungal colonies in the presence of the bacterium (Fig. 6A). The percentage of mycelial growth inhibition varied among 63% (for *F. graminearum*) and 38% (for *F. solani*). In agreement with these results, the addition of CFS from *B. ambifaria* T16 into the PDA medium at different concentrations, significantly inhibited the growth of *F. solani*, *F. oxysporum* and *F. graminearum* (Fig. 6B), reaching 69%, 57% and 66% of growth inhibition, respectively, when the CFS was added at a final concentration of 50% (v/v). Moreover, we also tested if the growth of *B. ambifaria* T16 at acid pH influenced growth inhibition of *F. oxysporum*. A similar inhibitory activity against *F. oxysporum* was observed with the CFS from cultures grown at pH 5.3 and 7.3 (Fig. 6C), with percentages of mycelial growth inhibition around 73% in both cases, suggesting that pH does not affect production of antifungal compounds in *B. ambifaria* T16.

4. Discussion

Biological control is a promising strategy to reduce or mitigate the negative effects of phytopathogenic *Fusarium* spp. and their mycotoxins on crops. FA is a secondary metabolite produced by several phytopathogenic *Fusarium* species, including *F. oxysporum*, *F. solani* and *F. verticillioides* (Pegg, 1981), which is toxic to plants (Chakrabarti and

Table 1
MIC of different antibiotics in *B. ambifaria* T16 growing in LB medium at different pHs.

Antibiotic	MIC in LB pH 5.3 (μg ml^{-1})	MIC in LB pH 7.3 (μg ml^{-1})
Potassium Tellurite	10	10
Cefotaxime	800	50
Ciprofloxacin	100	12.5
Sulfamethoxazole-trimethoprim	40	5

Chaudhary, 1980; Chakrabarti and Ghosal, 1989; Marrè et al., 1993; Pavlovkin et al., 2004; Singh and Upadhyay, 2014), mammalian cells (Fernandez-Pol et al., 1993; Ramautar, 2003), and bacteria (Bacon et al., 2006; Ruiz et al., 2015). In particular, *F. oxysporum* is responsible for vascular wilt diseases of an enormous variety of plant species (Bosland, 1998) and FA has been reported to play a very important role in plant pathogenesis (Pegg, 1981; El-Hassan et al., 2007). The universal presence of *F. oxysporum* in soils and the negative effects of FA on bacterial biocontrol activity (Duffy and Défago, 1997; Notz et al., 2002; van Rij et al., 2004; Quecine et al., 2015), suggest that the ability to degrade FA is a relevant and desirable characteristic to improve biocontrol effectiveness of *Fusarium* wilt.

The only records about the isolation of bacteria able to detoxify FA were reported by Toyoda et al. (1991) and Utsumi et al. (1991). Toyoda et al. isolated a *Klebsiella oxytoca* strain able to tolerate high concentrations of FA (up to 2.5 mM) and demonstrated FA detoxification by examining the survival of tomatos callus cells after the incubation with culture filtrates grown in the presence of FA. Utsumi et al. isolated a *Burkholderia cepacia* strain able to grow in minimal medium with FA as sole C source, but growth of this strain in liquid medium and degradation of the mycotoxin were not analyzed. In the present study, we described the isolation and characterization of *Burkholderia ambifaria* T16, a rhizospheric bacterial strain able to use FA as sole C, N and energy source. The phenotypic and biochemical characteristics of *B. ambifaria* T16 were similar to those described for other strains of the same species (Coenye et al., 2001). However, the ability to degrade FA has not been described in others *B. ambifaria* strains, so far.

B. ambifaria has been isolated from the rhizosphere of various plant species including pea, sugarcane and maize (Compant et al., 2008) and is one of the most abundant species of the *B. cepacia* complex (Bcc) found in agricultural soils and in the rhizosphere of several crops (Coenye and Vandamme, 2003). Due to their remarkable metabolic versatility, *Burkholderia* spp. strains have a great potential for biotechnological applications in plant growth promotion, biological control of important soil-borne phytopathogenic fungi and nematodes and bioremediation of diverse aliphatic and aromatic pollutants (Kilbane et al., 1982; Chiarini et al., 2006; Seo et al., 2007; Krastanov et al., 2013; Kang and Doty, 2014; Parra-Cota et al., 2014; Castanheira et al., 2016; Depoorter et al., 2016). The ability of *B. ambifaria* T16 to use FA, either as sole C or N source, was evaluated in liquid cultures. In both growth conditions, a prolonged lag phase was observed, which may be attributed to an adaptation phase, probably involving the activation of mechanisms that allow this bacterium, not only to degrade FA, but also

Table 2
Effect of *B. ambifaria* T16 on growth, yield and grain quality of barley plants under greenhouse conditions. Data are means from four replicates ± standard deviations (*P < 0.05).

	Control plants	T16-inoculated plants
Plant aerial biomass (g DW pot^{-1})	86.71 ± 3.07	92.38 ± 3.93 (P = 0.0637)
Spike dry weight (g DW pot^{-1})	49.98 ± 2.00	53.69 ± 2.05*
Spike number (pot^{-1})	47.75 ± 2.06	53.33 ± 3.21*
Grain yield (g pot^{-1})	43.83 ± 1.50	47.33 ± 1.90*
Grain size (fraction > 2.5 mm) (%)	98.43 ± 0.37	95.69 ± 2.00*
Grain N (mg N g^{-1}DW)	11.35 ± 0.54	10.08 ± 1.03 (P = 0.0731)

to tolerate the harmful effects reported for this mycotoxin (Fernandez-Pol et al., 1993; Marrè et al., 1993; Ramautar, 2003; Pavlovkin et al., 2004; Bacon et al., 2006; Ruiz et al., 2015). In other bacteria able to degrade toxic compounds, like cypermethrin and butachlor, an extended lag phase has also been observed (Dwivedi et al., 2010; Jilani, 2013). Moreover, *B. ambifaria* T16 grows faster when using FA as C source and ammonium chloride as N source than when using glucose as C source and FA as N source. A decrease of the initial FA content was observed along with bacterial growth, and the complete consumption of this compound coincided with the entry of the cultures into the stationary growth phase. Besides, according to our FA detoxification bioassays, *B. ambifaria* T16 was also able to degrade FA *in planta*, showing the ability to detoxify FA in barley seedlings, which was demonstrated by the reduction of the negative effects of FA on seedlings development.

Since *B. ambifaria* T16 was isolated from barley plants growing in acidic soil, we decided to evaluate the influence of pH on different characteristics of this bacterium. In LB medium adjusted to pH 5.3, *B. ambifaria* T16 showed a higher growth rate, higher cell densities and longer survival than the observed in LB adjusted to pH 7.3. These results are in agreement with a previous study that demonstrated that low pH tolerance is a general property of the genus *Burkholderia*, enabling them to colonize and survive in low pH environments (Stopnisek et al., 2014). Moreover, the resistance to cefotaxime, ciprofloxacin and sulfamethoxazole-trimethoprim was higher when *B. ambifaria* T16 was cultured at acid pH in comparison with neutral pH. As antibiotics stability is not affected at the examined pH range, and considering that pH alters the cell surface, the increased resistance to these compounds at acid pH could be due to changes in antibiotic permeability as proposed for *Salmonella* spp. (Laub et al., 1989). The proportion of surface-attached bacteria (biofilm formation) increased when *B. ambifaria* T16 was cultured in LB at pH 5.3 as compared to pH 7.3, in contrast to the observed by Ramli et al. (2012) in *B. pseudomallei*, in which enhanced biofilm formation was observed at neutral pH. Finally, higher levels of auxin production were observed when *B. ambifaria* T16 was grown at acid pH. While analyzing the factors affecting IAA production in *Azospirillum brasilense*, Ona et al. (2013) found that the expression of *ipdC*, the key gene for IAA production, was enhanced at low pH. To our knowledge, the effect of pH on IAA production in *Burkholderia* spp. has

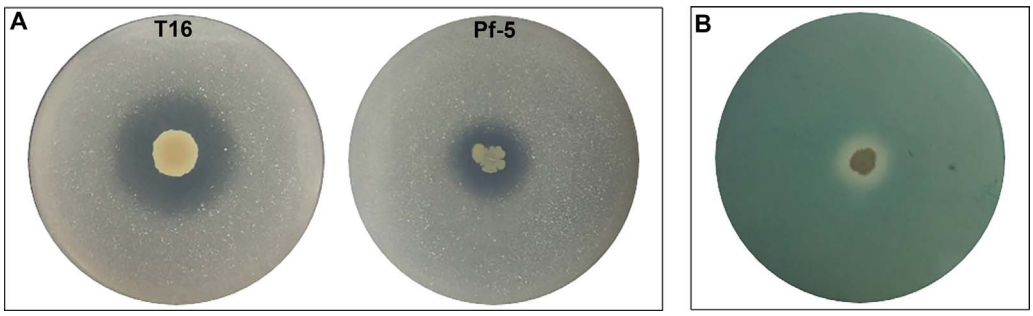

Fig. 5. Plant growth promoting traits of *B. ambifaria* T16. (**A**) Phosphate solubilization activity tested by plate assay using Pikovskaya agar medium. *P. protegens* Pf-5 was used as positive control. (**B**) Siderophore production assessed through the O-CAS assay.

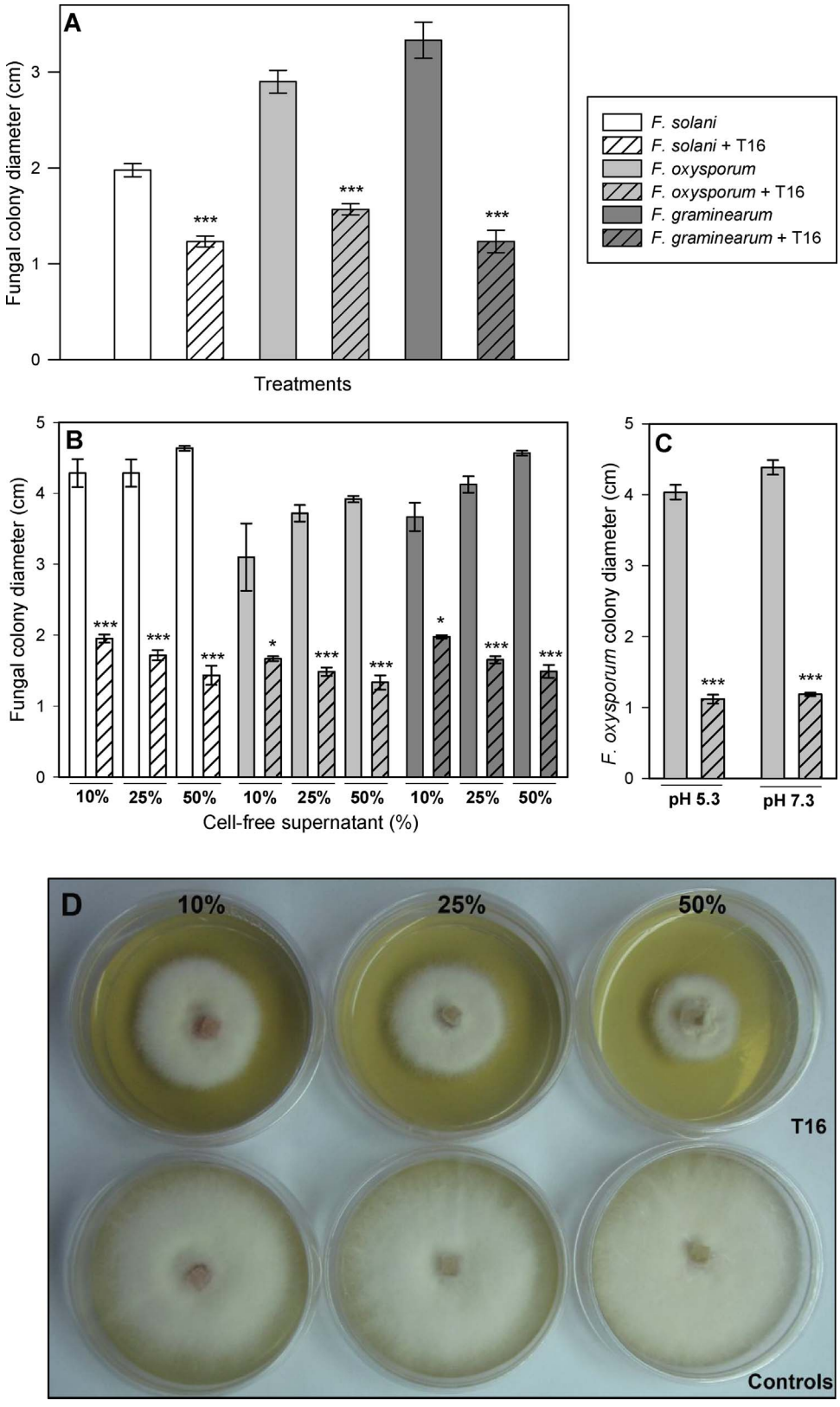

Fig. 6. (**A**) Antagonism of *B. ambifaria* T16 against *Fusarium* spp. tested by dual plate assays. Data represent the means of the colony diameter of each fungus in the presence (+T16) and in the absence of the bacterium ± standard deviations (n = 3). (**B**) Mycelial growth of *Fusarium* spp. in PDA containing different concentrations of cell-free supernatant (CFS) of *B. ambifaria* T16 or controls containing LB broth instead of CFS. Data are means from three replicates ± standard deviations. (**C**) Mycelial growth of *F. oxysporum* in PDA containing CFS from bacterial cultures grown in LB buffered at pH 5.3 and pH 7.3 or controls containing LB broth instead of CFS. The CFS was used at a concentration of 50%. Data are means from three replicates ± standard deviations. **A**, **B** and **C**: Significant differences between mean values are indicated by asterisks *P < 0.05, ***P < 0.001. (**D**) Photographic image showing the mycelial growth of *F. oxysporum* in PDA containing different concentrations of CFS from *B. ambifaria* and controls containing LB instead of CFS.

not been previously evaluated. It is worth mention that besides its role as a phytohormone, IAA was shown to be involved in the attenuation of potato tuber lesions caused by *Fusarium solani* f. sp. *eumartii* (Wang et al., 2016), highlighting the role of this molecule in bacterial-fungal interactions. All these results demonstrated that acid pH favored growth, survival, biofilm formation and resistance to antibiotics in *B. ambifaria* T16, thus contributing to the selective advantage of this strain in low pH environments.

Besides IAA production, *B. ambifaria* T16 exhibited other plant growth promoting traits, like siderophore production, phosphate solubilization and ACC deaminase activity. It is known that ACC activity improves plant nutrition by increasing ammonium availability in the

rhizosphere and also reduces ethylene levels in plants, therefore contributing to increase the resistance of plants to some environmental stresses. ACC deaminase activity seems to be a widespread feature in the genera *Burkholderia* and *Paraburkholderia* (Onofre-Lemus et al., 2009; Parra-Cota et al., 2014).

In greenhouse trials, barley plants inoculated with *B. ambifaria* T16 showed an increase in grain yield of about 8%. This increment was due to the higher number of spikes developed by inoculated plants resulting in more grains per pot, even though the average individual spike weight (g spike^{-1}) did not vary (not shown). Regarding grain quality, plants inoculated with *B. ambifaria* T16 showed a lower grain size (fraction > 2.5 mm) and a slight tendency to reduction in grain N content. It has been previously shown that the increase in the number of tillers leads to a reduction in the number and size of grains due to differences in maturity between the main stem and tillers (Ellis and Marshall, 1998). Also, reduced grain size has been reported in field-grown barley plants showing higher yields due to N fertilization (Magliano et al., 2014). Other authors have shown increased grain yield and harvest index in two grain amaranth cultivars inoculated with *B. ambifaria* Mex-5 and *B. caribensis* XV (Parra-Cota et al., 2014). These effects were observed not only under limited nutrient availability in soil but also after chemical fertilization with nitrogen and phosphorus.

B. ambifaria is known to suppress several soil-borne plant diseases caused by important fungal pathogens, such as *F. oxysporum*, *Phytium ultimum*, *P. aphanidermatum, Rhizotocnia solani*, *Sclerotium rolfsii*, *Alternaria alternata* and *Aphanomyces euteiches* (King and Parke, 1993; Mao et al., 1998; Li et al., 2002; Schmidt et al., 2009; Groenhagen et al.,2013). Pyrrolnitrin (Schmidt et al., 2009), burkholdines (Tawfik et al., 2010) and several volatile compounds (Groenhagen et al., 2013) are some of the metabolites that have been identified as responsible for fungal growth inhibition. The *in vitro* antagonism assays carried out in this study indicate that *B. ambifaria* T16 can inhibit the growth of several *Fusarium* species. Moreover, the antifungal activity detected in the cell-free supernatant, confirmed the production of extracellular compounds with antifungal activity in this strain. According to our results, fungal inhibition was not significantly different when *B. ambifaria* T16 was cultured in LB medium at pH 5.3 and 7.3, suggesting that the production of antifungal compounds was not affected by this range of pH under the tested conditions.

5. Conclusions

Burkholderia ambifaria T16 is able to inhibit the growth of several *Fusarium* species and possess the unique ability to use the mycotoxin fusaric acid as sole C, N and energy source, as well as the capacity to detoxify FA in barley seedlings. Moreover, inoculation of barley plants with this bacterium increased grain yield. The analysis of different characteristics of *B. ambifaria* T16 related to survival and plant growth promotion at neutral and acid pH demonstrated that is better adapted to acidic pH environments. *B. ambifaria* T16 can be utilized as a model system to study the physiology and genetics of FA tolerance and degradation and becomes a new source of metabolites or genes for the development of novel FA-detoxification systems.

Funding

This work was supported by grants from the Argentinean National Scientific and Technical Research Council (CONICET-PIP N°0255), University of Buenos Aires (UBACyT 20020130200117BA) and the Alexander von Humboldt Foundation.

Acknowledgments

We are grateful to Sebastián Stenglein and Mariela Echeverría for providing *Fusarium* spp. strains and Claudio Valverde for *P. protegens* Pf-5.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Bacon, C.W., Hinton, D.M., 1996. Fusaric acid and pathogenic interactions of corn and non-corn isolates of *Fusarium moniliforme*, a nonobligate pathogen of corn. Adv. Exp. Med. Biol. 392, 175–191.
Bacon, C.W., Porter, J.K., Norred, W.P., 1995. Toxic interaction of fumonisin B1 and fusaric acid measured by injection into fertile chicken egg. Mycopathologia 129, 29–35.
Bacon, C.W., Yates, I.E., Hinton, D.M., Meredith, F., 2001. Biological control of *Fusarium moniliforme* in maize. Environ. Health Perspect. 109, 325–332.
Bacon, C.W., Hinton, D.M., Hinton, A., 2006. Growth-inhibiting effects of concentrations of fusaric acid on the growth of *Bacillus mojavensis* and other biocontrol *Bacillus* species. J. Appl. Microbiol. 100, 185–194.
Baethgen, W.E., Alley, M.M., 1989. A manual colorimetric procedure for measuring ammonium nitrogen in soil and plant kjeldahl digests. Commun. Soil Sci. Plant Anal. 20, 961–969.
Bernar, E.M., Ruiz, J.A., 2016. El ácido fusárico, micotoxina producida por Fusarium spp., afecta negativamente el crecimiento de Pseudomonas protegens Pf-5 mediante el secuestro de hierro y la producción de especies reactivas de oxígeno. Química Viva, vol. 15 (Number 3). http://www.quimicaviva.qb.fcen.uba.ar.
Bosland, P.W., 1998. Fusarium oxysporum a pathogen of many plant species. Adv. Plant. Pathol. 6, 281–289.
Castanheira, N., Dourado, A.C., Kruz, S., Alves, P.I.L., Delgado-Rodríguez, A.I., Pais, I., Semedo, J., Scotti-Campos, P., Sánchez, C., Borges, N., Carvalho, G., Barreto Crespo, M.T., Fareleira, P., 2016. Plant growth-promoting *Burkholderia* species isolated from annual ryegrass in Portuguese soils. J. Appl. Microbiol. 120, 724–739.
Chakrabarti, D.K., Basu Chaudhary, K.C., 1980. Correlation between virulence and fusaric acid production in *Fusarium oxysporum* f. sp *carthami*. J. Phytopathol. 99, 43–46.
Chakrabarti, D.K., Ghosal, S., 1989. The disease cycle of mango malformation induced by *Fusarium moniliforme* var *subglutinans* and the curative effects of mangiferinmetal chelates. J. Phytopathol. 125, 128–146.
Chiarini, L., Bevivino, A., Dalmastri, C., Tabacchioni, S., Visca, P., 2006. Burkholderia cepacia complex species: health hazards and biotechnological potential. Trends Microbiol. 14, 277–286.
Chin-A-Woeng, T.F.C., Bloemberg, G.V., Bij, A.J.v.d., Drift, K.M.G.M.v.d., Schripsema, J., Kroon, B., Scheffer, R.J., Keel, C., Bakker, P.A.H.M., Tichy, H.-V., Bruijn, F.J.d., Thomas-Oates, J.E., Lugtenberg, B.J.J., 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp *radicis-lycopersici*. Mol. Plant-Microbe Interact. 11, 1069–1077.
Coenye, T., Vandamme, P., 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ. Microbiol. 5, 719–729.
Coenye, T., Mahenthiralingam, E., Henry, D., LiPuma, J.J., Laevens, S., Gillis, M., Speert, D.P., Vandamme, P., 2001. Burkholderia ambifaria sp. nov., a novel member of the Burkholderia cepacia complex including biocontrol and cystic fibrosis-related isolates. Int. J. Syst. Evol. Microbiol. 51, 1481–1490.
Compant, S., Nowak, J., Coenye, T., Clément, C., Ait Barka, E., 2008. Diversity and occurrence of *Burkholderia* spp. in the natural environment. FEMS Microbiol. Rev. 32, 607–626.
Depoorter, E., Bull, M.J., Peeters, C., Coenye, T., Vandamme, P., Mahenthiralingam, E., 2016. *Burkholderia*: an update on taxonomy and biotechnological potential as antibiotic producers. Appl. Microbiol. Biotechnol. 100, 5215–5229.
Duffy, B.K., Défago, G., 1997. Zinc improves biocontrol of *Fusarium* crown and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. Phytopathology 87, 1250–1275.
Duffy, B.K., Keel, C., Défago, G., 2004. Potential role of pathogen signaling in multitrophic plant-microbe interactions involved in disease protection. Appl. Environ. Microbiol. 70, 1836–1842.
Dwivedi, S., Singh, B.R., Al-Khedhairy, A.A., Alarifi, S., Musarrat, J., 2010. Isolation and characterization of butachlor-catabolizing bacterial strain *Stenotrophomonas acidaminiphila* JS-1 from soil and assessment of its biodegradation potential. Lett. Appl. Microbiol. 51, 54–60.
Dworkin, M., Foster, J.W., 1958. Experiments with some microorganism which utilize ethane and hydrogen. J. Bacteriol. 75, 592–603.
Edi Premono, J., Moawad, A.M., Vlek, P.L.G., 1996. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indones. J. Crop Sci. 11, 13–23.
El-Hassan, K.I., El-Saman, M.G., Mosa, A.A., Mostafa, M.H., 2007. Variation among *Fusarium* spp., the causal of potato tuber dry rot in their pathogenicity and mycotoxins production. Egypt. J. Phytopathol. 35, 53–68.
Ellis, R.P., Marshall, B., 1998. Growth, yield and grain quality of barley (*Hordeum vulgare* L.) in response to nitrogen uptake. II. Plant development and rate of germination. J. Exp. Bot. 49, 1021–1029.
Fernandez-Pol, J.A., Klos, D.J., Hamilton, P.D., 1993. Cytotoxic activity of fusaric acid on human adenocarcinoma cells in tissue culture. Anticancer Res. 13, 57–64.
Glickman, E., Dessaux, Y., 1995. A critical examination of the specificity of the Salkowski's reagent for indolic compounds produced by phytopathogenic bacteria. Appl. Environ. Microbiol. 61, 793–796.
Gould, W.D., Hagedorn, C., Bardinelli, T.R., Zablotowicz, R.M., 1985. New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. Appl. Environ. Microbiol. 49, 28.
Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S., Weisskopf,

L., 2013. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. J. Chem. Ecol. 39, 892–906.
Hagedorn, C., Gould, W.D., Bardinelli, T.R., Gustavson, D.R., 1987. A selective medium for enumeration and recovery of *Pseudomonas cepacia* biotypes from soil. Appl. Environ. Microbiol. 53, 2265–2268.
Howell, C., Stipanovic, R., 1979. Control of *Rhizoctonia solani* in cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69, 480–482.
Huisman, G.W., Wonink, E., de Koning, G.J.M., Preusting, H., Witholt, B., 1992. Synthesis of poly (3-hydroxyalkanoates) by mutant and recombinant *Pseudomonas* strains. Appl. Microbiol. Biotechnol. 38, 1–5.
Jilani, S., 2013. Comparative assessment of growth and biodegradation potential of soil isolate in the presence of pesticides. Saudi J. Biol. Sci. 20, 257–264.
Kang, J.W., Doty, S.L., 2014. Cometabolic degradation of trichloroethylene by *Burkholderia cepacia* G4 with poplar leaf homogenate. Can. J. Microbiol. 60, 487–490.
Kilbane, J.J., Chatterjee, D.K., Karns, J.S., Kellogg, S.T., Chakrabarty, A.M., 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. 44, 72–78.
King, E.B., Parke, J.L., 1993. Biocontrol of *Aphanomyces* root rot and *Phytium* damping off by *Pseudomonas cepacia* AMMD on four pea cultivars. Plant Dis. 77, 1185–1188.
Krastanov, A., Alexieva, Z., Yemendzhiev, H., 2013. Microbial degradation of phenol and phenolic derivatives. Eng. Life Sci. 13, 76–87.
Lageveen, R., Huisman, Preusting, G.W., Ketelaar, H., Eggink, P.G., Witholt, B., 1988. Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. Appl. Environ. Microbiol. 54, 2924–2932.
Laub, R., Schneider, Y.J., Trouet, A., 1989. Antibiotic susceptibility of *Salmonella* spp. at different pH values. J. Gen. Microbiol. 135, 1407–1416.
Li, W., Roberts, D.P., Dery, P.D., Meyer, S.L.F., Lohrke, S., Lumsden, R.D., Hebbar, K.P., 2002. Broad spectrum antibiotic activity and disease suppression by the potential biocontrol agent *Burkholderia ambifaria* BC-F. Crop Prot. 21, 129–135.
Li, C., Zuo, C., Deng, G., Kuang, R., Yang, Q., Hu, C., Sheng, O., Zhang, S., Ma, L., Wei, Y., Yang, J., Liu, S., Biswas, M.K., Viljoen, A., Yi, G., 2013. Contamination of bananas with beauvericin and fusaric acid produced by *Fusarium oxysporum* f. sp *cubense*. PLoS One 8, e70226.
Long, H.H., Sonntag, D.G., Schmidt, D.D., Baldwin, I.T., 2010. The structure of the culturable root bacterial endophyte community of Nicotiana attenuata is organized by soil composition and host plant ethylene production and perception. New Phytol. 185, 554–567.
Loper, J.E., Hassan, K.A., Mavrodi, D.V., Davis 2nd, E.W., Lim, C.K., Shaffer, B.T., Elbourne, L.D., Stockwell, V.O., Hartney, S.L., Breakwell, K., Henkels, M.D., Tetu, S.G., Rangel, L.I., Kidarsa, T.A., Wilson, N.L., van de Mortel, J.E., Song, C., Blumhagen, R., Radune, D., Hostetler, J.B., Brinkac, L.M., Durkin, A.S., Kluepfel, D.A., Wechter, W.P., Anderson, A.J., Kim, Y.C., Pierson 3rd, L.S., Pierson, E.A., Lindow, S.E., Kobayashi, D.Y., Raaijmakers, J.M., Weller, D.M., Thomashow, L.S., Allen, A.E., Paulsen, I.T., 2012. Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genet. 8, e1002784.
Magliano, P.N., Prystupa, P., Gútierrez-Boem, F.H., 2014. Protein content of grains of different size fraction in malting barley. J. Inst. Brew. 120, 347–352.
Mao, W., Lewis, J.A., Lumsden, R.D., Hebbar, K.P., 1998. Biocontrol of selected soilborne diseases of tomato and pepper plants. Crop Prot. 17, 535–542.
Marrè, M.T., Vergani, P., Albergoni, F.G., 1993. Relationship between fusaric acid uptake and its binding to cell structures by leaves of *Egeria densa* and its toxic effects on membrane permeability and respiration. Physiol. Mol. Plant Pathol. 42, 141–157.
Merritt, J.H., Kadouri, D.E., ÓToole, G.A., 2005. Growing and analyzing static biofilms. Curr. Protoc. Microbiol (Unit IB.1.1-1B.1.17).
Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
Notz, R., Maurhofer, M., Dubach, H., Haas, D., Defago, G., 2002. Fusaric acid-producing strains of *Fusarium oxysporum* alter 2, 4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 *in vitro* and in the rhizosphere of wheat. Appl. Environ. Microbiol. 68, 2229–2235.
Ona, O., Smets, I., Gysegom, P., Bernaerts, K., Van Impe, J., Prinsen, E., Vanderleyden, J., 2013. The effect of pH on indole-3-acetic acid (IAA) biosynthesis of *Azospirillum brasilense* Sp7. Symbiosis 35, 199–208.
Onofre-Lemus, J., Hernandez-Lucas, I., Girard, L., Caballero-Mellado, J., 2009. ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants. Appl. Environ. Microbiol. 75, 6581–6590.
Ostle, A., Holt, J.G., 1982. Nile Blue A as a fluorescent stain for poly-b-hydroxybutyrate. Appl. Environ. Microbiol. 44, 238–241.
Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L.S., Fernández, F.J., 2007. O-CAS, a fast and universal method for siderophore detection. J. Microbiol. Methods 70, 127–131.
Parra-Cota, F.I., Pena-Cabriales, J.J., de Los Santos-Villalobos, S., Martinez-Gallardo, N.A., Delano-Frier, J.P., 2014. *Burkholderia ambifaria* and B. *caribensis* promote growth and increase yield in grain amaranth (*Amaranthus cruentus* and *A. hypochondriacus*) by improving plant nitrogen uptake. PLoS One 9, e88094.
Pavlovkin, J., Mistrík, I., Prokop, M., 2004. Some aspects of the phytotoxic action of fusaric acid on primary *Ricinus* roots. Plant Soil Environ. 50, 397–401.
Payne, G.W., Vandamme, P., Morgan, S.H., Lipuma, J.J., Coenye, T., Weightman, A.J., Jones, T.H., Mahenthiralingam, E., 2005. Development of a *recA* gene-based identification approach for the entire *Burkholderia* genus. Appl. Environ. Microbiol. 71, 3917–3927.
Pegg, G.F., 1981. Biochemistry and physiology of pathogenesis. In: Mace, M.E., Bell, A.A., Beckman, C.H. (Eds.), Fungal Wilt Diseases of Plants. Academic Press Inc., New York, pp. 193–246.
Penrose, D.M., Glick, B.R., 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol. Plant 118, 10–15.
Pikovskaya, R.I., 1948. Mobilization of phosphorus in soil connection with vital capacity of source microbial *species*. Microbiologia 17, 362–370.
Quecine, M.C., Kidarsa, T.A., Goebel, N.C., Shaffer, B.T., Henkels, M.D., Zabriskie, T.M., Loper, J.E., 2015. An interspecies signaling system mediated by fusaric acid has parallel effects on antifungal metabolite production by *Pseudomonas protegens* Strain Pf-5 and antibiosis of *Fusarium* spp. Appl. Environ. Microbiol. 82, 1372–1382.
Ramautar, A., 2003. An Investigation into the Apoptotic Inducing Effect of Fusaric Acid on Human Lymphocytes and Its Role in Cell Growth Inhibition. Master Thesis. Department of Physiology.University of Natal.
Ramli, N.S., Eng Guan, C., Nathan, S., Vadivelu, J., 2012. The effect of environmental conditions on biofilm formation of *Burkholderia pseudomallei* clinical isolates. PLoS One 7, e44104.
Ruiz, J.A., Bernar, E., Jung, K., 2015. Production of siderophores increases resistance to fusaric acid in *Pseudomonas protegens* Pf-5. PLoS One 10, e0117040.
Saikia, R., Varghese, S., Singh, B.P., Arora, D.K., 2009. Influence of mineral amendment on disease suppressive activity of *Pseudomonas fluorescens* to *Fusarium* wilt of chickpea. Microbiol. Res. 164, 365–373.
Schmidt, S., Blom, J.F., Pernthaler, J., Berg, G., Baldwin, A., Mahenthiralingam, E., Eberl, L., 2009. Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the *Burkholderia cepacia* complex. Environ. Microbiol. 11, 1422–1437.
Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Defago, G., Haas, D., Keel, C., 2000. Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. J. Bacteriol. 182, 1215–1225.
Seo, J.S., Keum, Y.S., Hu, Y., Lee, S.E., Li, Q.X., 2007. Degradation of phenanthrene by *Burkholderia* sp. C3: initial 1, 2- and 3, 4-dioxygenation and meta- and ortho-cleavage of naphthalene-1, 2-diol. Biodegradation 18, 123–131.
Silvestro, L.B., Stenglein, S.A., Forjan, H., Dinolfo, M.I., Arambarri, A.M., Manso, L., Moreno, M.V., 2013. Occurrence and distribution of soil *Fusarium* species under wheat crop in zero tillage. Span. J. Agric. Res. 11, 72–79.
Singh, V.K., Upadhyay, R.S., 2014. Fusaric acid induced cell death and changes in oxidative metabolism of *Solanum lycopersicum L*. Bot. Stud. 55, 66.
Smith, T.K., 1992. Recent advances in the understanding of *Fusarium* trichothecene mycotoxicoses. J. Anim Sci. 70, 3989–3993.
Spilker, T., Baldwin, A., Bumford, A., Dowson, C.G., Mahenthiralingam, E., LiPuma, J.J., 2009. Expanded multilocus sequence typing for *Burkholderia* species. J. Clin. Microbiol. 47, 2607–2610.
Stopnisek, N., Bodenhausen, N., Frey, B., Fierer, N., Eberl, L., Weisskopf, L., 2014. Genus-wide acid tolerance accounts for the biogeographical distribution of soil *Burkholderia* populations. Environ. Microbiol. 16, 1503–1512.
Tabacchioni, S., Ferri, L., Manno, G., Mentasti, M., Cocchi, P., Campana, S., Ravenni, N., Taccetti, G., Dalmastri, C., Chiarini, L., Bevivino, A., Fani, R., 2008. Use of the *gyrB* gene to discriminate among species of the *Burkholderia cepacia* complex. FEMS Microbiol. Lett. 281, 175–182.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Tawfik, K.A., Jeffs, P., Bray, B., Dubay, G., Falkinham, J.O., Mesbah, M., Youssef, D., Khalifa, S., Schmidt, E.W., 2010. Burkholdines 1097 and 1229, potent antifungal peptides from *Burkholderia ambifaria* 2. 2N. Org. Lett. 12, 664–666.
Toyoda, H., Katsuragi, K.T., Tamai, T., Ouchi, S., 1991. DNA sequence of genes for detoxification of fusaric acid, a wilt-inducing agent produced by *Fusarium* species. J. Phytopathol. 133, 265–277.
Utsumi, R., Yagi, T., Katayama, S., Katsuragi, K., Tachibana, K., Toyoda, H., Ouchi, S., Obata, K., Shibano, Y., Noda, M., 1991. Molecular cloning and characterization of the fusaric acid-resistance gene from *Pseudomonas cepacia*. Agric. Biol. Chem. 7, 1913–1918.
Vanlaere, E., Lipuma, J.J., Baldwin, A., Henry, D., De Brandt, E., Mahenthiralingam, E., Speert, D., Dowson, C., Vandamme, P., 2008. *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex. Int. J. Syst. Evol. Microbiol. 58, 1580–1590.
Wang, M., Tachibana, S., Murai, Y., Li, L., Lau, S.Y.L., Cao, M., Zhu, G., Hashimoto, M., Hashidoko, Y., 2016. Indole-3-acetic acid produced by *Burkholderia heleia* acts as a phenylacetic acid antagonist to disrupt tropolone biosynthesis in *Burkholderia plantarii*. Sci. Rep. 6, 22596.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
Weller, D.M., Raaijmakers, J.M., Gardener, B.B., Thomashow, L.S., 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu. Rev. Phytopathol. 40, 309–348.
van Rij, E.T., Wesselink, M., Chin, A., Woeng, T.F., Bloemberg, G.V., Lugtenberg, B.J., 2004. Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. Mol. Plant Microbe Interact. 17, 557–566.
van Rij, E.T., Girard, G., Lugtenberg, B.J., Bloemberg, G.V., 2005. Influence of fusaric acid on phenazine-1-carboxamide synthesis and gene expression of Pseudomonas chlororaphis strain PCL1391. Microbiology 151, 2805–2814.