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# Phenotypic traits of *Burkholderia* spp. associated with ecological adaptation and plant-host interaction

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ABSTRACT

*Burkholderia* species have different lifestyles establishing mutualist or pathogenic associations with plants and animals. Changes in the ecological behavior of these bacteria may depend on genetic variations in response to niche adaptation. Here, we studied 15 *Burkholderia* strains isolated from different environments with respect to genetic and phenotypic traits. By Multilocus Sequence Analysis (MLSA) these isolates fell into 6 distinct groups. MLSA clusters did not correlate with strain antibiotic sensitivity, but with the bacterial ability to produce antimicrobial compounds and control orchid necrosis. Further, the *B. seminalis* strain TC3.4.2R3, a mutualistic bacterium, was inoculated into orchid plants and the interaction with the host was evaluated by analyzing the plant response and the bacterial oxidative stress response *in planta*. TC3.4.2R3 responded to plant colonization by increasing its own growth rate and by differential gene regulation upon oxidative stress caused by the plant, while reducing the plant's membrane lipid peroxidation. The bacterial responses to oxidative stress were recapitulated by bacterial exposure to the herbicide paraquat. We suggest that the ability of *Burkholderia* species to successfully establish in the rhizosphere correlates with genetic variation, whereas traits associated with antibiotic resistance are more likely to be categorized as strain specific.

## 1. Introduction

*Burkholderia* species are often found in association with plants in the endosphere, phyllosphere and rhizosphere (via nodulation or free-living lifestyles) (Eberl and Vandamme, 2016). In addition, several *Burkholderia* spp. have been reported to establish antagonistic (via production of potent antifungal compounds) or mutualistic interactions with distinct soil fungi; e.g. *B. terrae* (Warmink and van Elsas, 2009) and *B. rhizoxinica* (Partida-Martinez and Hertweck, 2005); whereas others can be endosymbionts in insects (Kikuchi et al., 2005). In clinical settings, genetically distinct but phenotypically similar *Burkholderia* spp. comprising the *Burkholderia cepacia* complex (Bcc) have been isolated from chronic infection in immunocompromised patients, especially chronic cystic fibrosis patients, emerging as an opportunistic pathogen that causes severe infection (Mahenthiralingam et al., 2005; Eberl and Vandamme, 2016). In some vein, genetically similar strains can be isolated from soil or immunocompromised patients.

Species of the *Burkholderia* genus have well-known biotechnological potential, which include beneficial effects as antagonists to phytopathogens and plant growth promoters (Estrada-De Los Santos et al., 2001; Perin et al., 2006; Dourado et al., 2013; Araújo et al., 2016). Members of this genus are often capable of nitrogen fixation, phosphorus solubilization, and xenobiotic catabolism (Coenye et al., 2001; Minerdi et al., 2001; O'Sullivan and Mahenthiralingam, 2005; Vu et al., 2013; Shehata et al., 2016). However, the potential to cause disease in humans, especially the immunocompromised, has hampered the generalized use of *Burkholderia* species as biocontrol agents. Recently, Eberl and Vandamme (2016) identified two main clades within this genus, which were consistent with a pathogenic group and an environmental/plant beneficial group. The latter encompasses several species of environmental *Burkholderia* beneficial for plants. The clade of pathogenic strains for humans, animals, and plants include *B. pseudomallei, B. mallei*

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and *B. glumae*, and the species of the Bcc.

However, the Bcc also includes species that are known as plant growth promoters and biological control agents, such as *B. vietnamiensis, B. ambifaria* (Parke and Gurian-Sherman, 2001) and *B. cenocepacia* (Chávez-Ramírez et al., 2020). Other species tolerate heavy metals such as cadmium (Abou-Shanab et al., 2007), and, in general, members within the Bcc exhibit high levels of metabolic diversity, being potentially useful as herbicides and for bioremediation of contaminated soils (Coenye et al., 2001; Coenye and Vandamme, 2003). Despite being originally described as a phytopathogen (Li et al., 2010), and isolated from cystic fibrosis patients (Zhu et al., 2016), *B. seminalis* is an example of a Bcc species that can effectively control the phytopathogenic *B. gladioli* in orchid (Araújo et al., 2016). This phytopathogenic bacterium also causes tobacco and rice leaf necrosis (Furuya et al., 1997), internal corn straw rot (Lu et al., 2007), rice panicle rust (Fiori et al., 2011), and is an opportunistic human pathogen (Dursun et al., 2012). Therefore, it is clear that the differentiation between beneficial and pathogenic *Burkholderia* is context-dependent, and as such, cannot be inferred solely based on genetic information.

The ability of a bacterium to colonize distinct environments, such as soil, animals, and plants, depends on genes that are differentially regulated in an environmental specific manner. Also, shifts in the local environment may result in changes in the nature of an ecological interaction, e.g. from mutualistic to pathogenic lifestyles. For instance, the virulence of *B. seminalis* towards *Galleria mellonella* is manifested at 37 °C, while the ability to inhibit phytopathogenic fungi was induced at 28 °C (Gonçalves et al., 2019). This result suggests that the temperature could regulate the virulence in an animal model (37 °C) or the capability to inhibit fungi in the rhizosphere (28 °C). Moreover, the role of DNA methylation and genomic islands in the regulation of iron, trehalose and D-arabitol utilization operons was proposed as an evolutionary signature in the adaptation of *B. seminalis* strains isolated from different environments, such as cystic fibrosis sputum, water, soil, and apricot (Zhu et al., 2016). Therefore, the ecological adaptation of *Burkholderia* may result from strain-specific metabolic features and differential regulation of operons associated with specific niches (Zhu et al., 2016).

In this study, we characterized a group of *Burkholderia* spp. isolated from different environments. to identify correlations between genetic profile (using multilocus sequencing analysis, MLSA) with specific traits (orchid necrosis control, enzyme and antimicrobial production) and host-pathogen interaction (virulence to *Galleria mellonella* and antibiotic resistance). The group of isolates we investigated also included *B. seminalis* TC3.4.2R3, which was cultured from internal sugarcane root tissues (Luvizotto et al., 2010) and was able to control orchid necrosis (Araújo et al., 2016). Our results revealed positive correlations between the genetic profile and phenotypic traits. In contrast, the antibiotic resistance profile did not correlate with the genetic profile, suggesting that antibiotic resistance gene pools could be acquired by horizontal gene transfer. We also identified genes in *B. seminalis* TC3.4.2R3 associated with antibiotic resistance and investigated in more detail the interaction of this endophytic bacterium with orchid plants.

## 2. Material and methods

### 2.1. Burkholderia spp. strains and growth, and plant material

The *Burkholderia* spp. strains used in this study were obtained mostly from environmental samples, including soil, rhizosphere and roots, as well as isolates obtained from the different parts of the sugarcane plant (*Saccharum* spp.) (for a detailed description see Table 1). In general, independent cultures of each strain were grown in tryptic soy broth (TSB) at 28 °C for 24 h in a shaker incubator (150 rpm). To determine growth curves, these cultures were grown as pre-inoculum to an initial optical density ($OD_{600nm}$) of 0.04 ($8 \times 10^6$ CFU ml $^{-1}$) in 5% TSB medium. Cultures were incubated in the BioTek plate reader using 425 rpm at 28 °C, and the growth was monitored every 2 h during a total of 32 h. These assays were performed for each individual isolate using a total of 4 replicates.

**Table 1**
Description of the *Burkholderia* sp. strains used in this study.

| Strain | Species | Isolation place | References |
|---|---|---|---|
| CV3.2.2F5 | *Burkholderia* sp. | Root endophyte | Luvizotto et al. (2010) |
| TC3.4.1R1 | *Burkholderia* sp. | Root endophyte | Luvizotto et al. (2010) |
| TH3.3.2F5 | *Burkholderia* sp. | Rhizosphere | LABMEM, Department of Microbiology, ICB/USP |
| CV3.3.3F2 | *Burkholderia* sp. | Rhizosphere | Luvizotto et al. (2010) |
| TC3.4.2R2 | *Burkholderia* sp. | Root endophyte | Luvizotto et al. (2010), Araújo et al. (2016) |
| TC3.3.3F1 | *Burkholderia* sp. | Rhizosphere | Luvizotto et al. (2010) |
| TC3.4.1F2 | *Burkholderia* sp. | Root endophyte | Luvizotto et al. (2010) |
| 67SI | *Burkholderia* sp. | Soil | Gonçalves (2007) |
| 93Rz | *Burkholderia* sp. | Rhizosphere | Gonçalves (2007) |
| TC3.4.2R3 | *B. seminalis* | Root endophyte | Luvizotto et al. (2010) |
| AN 5.5 | *Burkholderia* sp. | Antarctic isolate | Bioproducts laboratory collection. ICB. USP |
| 28Rz | *Burkholderia* sp. | Rhizosphere | Gonçalves (2007) |
| 47Rz | *Burkholderia* sp. | Rhizosphere | Gonçalves (2007) |
| CMAA 1233 | *Burkholderia* sp. | Plant | EMBRAPA Jaguariuna Collection |
| LMG 2216 (type strain) | *B. gladioli* | Plant pathogen | Institut für Pflanzenbiologie der Universität Zürich collection |

For the plant assays, orchids (*Oncidium* Alowa Iwanaga, a hybrid between *Oncidium* goldiana and *Oncidium* Star Wars) were obtained from "Green Plugs Mudas de Flores e Plantas" (Mogi das Cruzes, São Paulo, Brazil).

### 2.2. Multilocus Sequence Analysis (MLSA)

Multilocus Sequence Analysis (MLSA) was performed using the *atpD* (ATP synthase β subunit), *gltB* (short-chain glutamate synthase), and *gyrB* (DNA gyrase, B subunit) genes to obtain phylogenetic information on the *Burkholderia* isolates. We used the primer sets previously described by Spilker et al. (2009), under the following conditions: initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 30 s, annealing temperature of each primer pair at 53 °C, 55 °C and 53 °C (for the *gltB*, *atpD* and *gyrB* gene, respectively) for 30 s, extension at 72 °C for 90 s, and a final extension step at 72 °C for 7 min. Amplifications were performed in a final volume of 50 µl containing 12.5 µl of EasyTaq® DNA Polymerase, 2.0 µl (5 − 20 ng. ml$^{-1}$) DNA, 10 pmol of each primer, and 33.5 µl milli-Q water. PCR amplicons were checked by electrophoresis, purified using a polyethylene glycol method (PEG 800020 %; NaCl 2.5 mM), and sequenced at the Center for Human Genome Studies, Institute of Biology, University of São Paulo. Sequences were analyzed using BLASTn (National Center Biotechnology Information, NCBI) against the GenBank database. Phylogenetic reconstructions were performed using the Neighbor Joining Method (NJ) method with 1000 replicates based on the genetic distance matrices calculated by the Jukes-Cantor model (1969), using MEGA v. 6.

### 2.3. Antagonism assays against pathogenic bacteria and fungi

The antagonistic assays were performed using the overlay method. The *Burkholderia* spp. isolates were cultured in TSB medium for 24 h at 28 °C. Ten-µl aliquots were inoculated onto Petri dishes containing TSB agar medium at 28 °C for two days, allowing the initial growth of *Burkholderia* spp. and the diffusion of bacterial metabolites in the medium. After that, *Burkholderia* spp. colonies were inactivated by exposure to UV radiation for 1 h. Pathogenic bacteria (*Escherichia coli, Bacillus* sp., *Staphylococcus aureus* and multi-resistant *Pseudomonas aeruginosa*) obtained from the LABMEM/NAP-BIOP (Department of

Microbiology, ICB/USP) collection were grown in TSB for 24 h at 28 °C with shaking (150 rpm). Then, 5-ml overlay of semi-solid TSB culture medium containing 100 µl of pathogenic bacterial culture was added over the inactive colonies. These flasks were incubated at 28 °C for 48 h, after which the presence of potential inhibition halos was recorded.

For antifungal activity, 10 µl aliquots of *Burkholderia* spp. grown as described above were inoculated onto Petri dish plates with PDA medium, and incubated for two days at 28 °C. After growth, 5 mm diameter disks of PDA medium containing the phytopathogenic fungi (*Ceratocystis paradoxa, Fusarium verticillioides, Aspergillus fumigatus, Colletotrichum* sp., and *Ceratocystis fimbriata*) mycelium were deposited on the surface of the plates in an opposite side as that of the *Burkholderia* spp. isolate. As controls, disks of each fungus were tested on plates containing only the PDA medium. Each individual *Burkholderia* spp. isolate was also grown on PDA to test cell viability and growth. Plates were incubated at 28 °C for 5 days, and the inhibition halos were evaluated. Each treatment (for both antibacterial and antifungal antagonism assays) contained a total of 6 replicates.

### 2.4. Phosphate solubilization, and siderophore and cellulase production

The ability of these strains to solubilize inorganic phosphate was quantitatively evaluated by measuring the halo obtained after bacterial growth on a medium supplemented with $Ca_3(PO_4)_2$. Plates were scored after seven days of incubation at 28 °C (Verma et al., 2001). Siderophore production was assayed according to Schwyn and Neilands (1987), using Chromo Azurol S Agar (CAS). A yellow or orange halo around the bacterial colony indicated a positive result for siderophore production. For endoglucanase detection, the bacteria were grown on M9 minimal medium (Sigma) plates containing 0.5 % yeast extract and 1 % Carboxymethylcellulose (CMC) (*w/v*). After microbial growth, 10 ml 0.1 % Congo Red solution were added, incubated at room temperature for 15 min and washed with NaCl (5 M). The presence of a colorless or yellowish halo surrounding the colony indicated the activity of endoglucanase (Teather and Wood, 1982).

### 2.5. Biocontrol of orchid necrosis and interaction with the host plant

This assay was performed as described in Araújo et al. (2016). Briefly, *Burkholderia* spp. strains were grown, washed with PBS buffer, and resuspended to a cell density of $10^5$ CFU $ml^{-1}$. To evaluate the suppressive potential of the strains, orchid leaves were punctured with a sterile toothpick and 5 µl of cell suspensions at $OD_{600nm}$ = 1.0 were placed on the resulting wound. All *Burkholderia* spp. were co-inoculated with *B. gladioli* LMG 2216. *B. gladioli* LMG 2216 and PBS were inoculated alone as positive and negative controls, respectively. Three biological replicas were carried out per strain. The plants were kept at 25 °C with 85 % relative humidity for 5 days. Each individual plant was visually inspected every day for the presence of necrosis around the *B. gladioli* inoculation point.

The beneficial interaction between a *Burkholderia* spp. with orchid plants was examined using the *B. seminalis* strain TC3.4.2R3 in experiments assessing the bacterium and plant responses. The strain TC3.4.2R3 was previously showed to effectively control Orchid necrosis (Araújo et al., 2016), to inhibit specific fungi, and had no virulence in mice (Gonçalves et al., 2019). For the assay, *B. seminalis* TC3.4.2R3 was inoculated into leaves and pseudobulbs with or without *B. gladioli* and the bacteria and plant responses were monitored for the presence of necrose and further evaluated. In addition, to assess the potential suppressive effect of *B. seminalis* against *B. gladioli,* both bacteria were inoculated by infiltration in abaxial leaf tissues. For this, we inoculated *B. seminalis* and incubated for 6 days to guarantee endophytic colonization. After this period, *B. gladioli* was inoculated using the same strategy 2 cm apart from the endophytic strain. The presence of symptoms was evaluated every day for 10 days.

### 2.6. Animal model using Galleria mellonella larvae

The *Burkholderia* spp. strains were grown in TSB for 24 h at 28 °C. Cultures with an initial $OD_{600}$ of 1.0 were centrifuged at 13,000 rpm for 5 min and resuspended in PBS. Ten-µl aliquots of each bacterial strain were applied to the pro-leg of fifth to sixth instar stage (approximately between 2 and 2.5 cm in length) *G. mellonella* larvae (250 − 300 mg). Inoculated larvae were maintained in Petri dishes at 28 °C. Uninoculated larvae and larvae inoculated with PBS were used as negative controls. Twenty-µl of the dilution $10^6$ and $10^7$ of each inoculum were seeded onto TSB plates to confirm the concentration and purity of the inoculum. *G. mellonella* mortality was evaluated every day for 7 days. The inoculation of bacteria into *G. mellonella* was carried out in triplicate, each replicate containing a total of 10 larvae (Pereira et al., 2015).

### 2.7. Antibiotic resistance

Antibiotic resistance was tested by direct inoculating the *Burkholderia* spp. isolates in saline solution with an $OD_{600}$ of 0.5 using the turbidity standard of the McFarland scale. A sterile cotton swab was dipped into the suspension of the standardized culture and spread evenly over the surface of Mueller-Hinton agar. Plates were allowed to dry, and individual antibiotic disks (Cefar Diagnóstica Ltda, Brazil) (Table 2) were placed in each plate. Cultures were incubated at 28 °C for 24 h and the diameters of the growth inhibition haloes were measured.

### 2.8. Evaluating antibiotic resistance genes potentially acquired via horizontal gene transfer

The genome of the strain TC3.4.2R3 was used as a model organism to identify genes associated with antibiotic resistance potentially acquired via horizontal gene transfer. The CARD (Comprehensive Antibiotic Resistance Database) database was used to perform qualitative prediction of genes conferring resistance to antibiotics. This database includes 4094 Ontology Terms and 2570 reference sequences (Jia et al., 2017). The complete genome of *B. seminalis* TC3.4.2R3was analyzed against CARD using BLASTx. Positive genes were inferred with a similarity threshold of ≥60 % and an *E*-value cutoff of $10^{-5}$.

The genomic island locations were predicted using the Alien Hunter software (Vernikos and Parkhill, 2006). The presence of genes that confer resistance to antibiotics in regions of genomic islands was performed manually using the results obtained by Alien Hunter and

**Table 2**
Description of antimicrobial compounds and respective disc concentrations used in this study.

| Class | Antimicrobial | Disc concentration (µg/ml) |
|---|---|---|
| Sulfonamides | Sulfamethoxazole-Trimethoprim (SZT) | 25 |
| Amphenicol | Chloramphenicol (CHL) | 30 |
| Carbapenem | Imipenem (IPM) | 10 |
| | Meropenem (MEM) | 10 |
| Cephalosporin | Ceftazidime (CAZ) | 30 |
| | Cefepime (FEP) | 30 |
| fluoroquinolone | Levofloxacin (LVX) | 5 |
| Beta-lactams | Piperacillin (PIP) | 20 |
| | Piperacillin + Tazobactam (TZP) | 110 |
| | Ticarcillin + Clavulanate (TIC) | 85 |
| | Carbenicillin (CAR) | 100 |
| monobactams | Aztreonam (ATM) | 30 |
| Glycylcycline | Tigecycline (TGC) | 15 |
| Tetracycline | Minocycline (MIN) | 30 |
| | Doxycycline (DOX) | 30 |
| | Tetracycline (TET) | 30 |

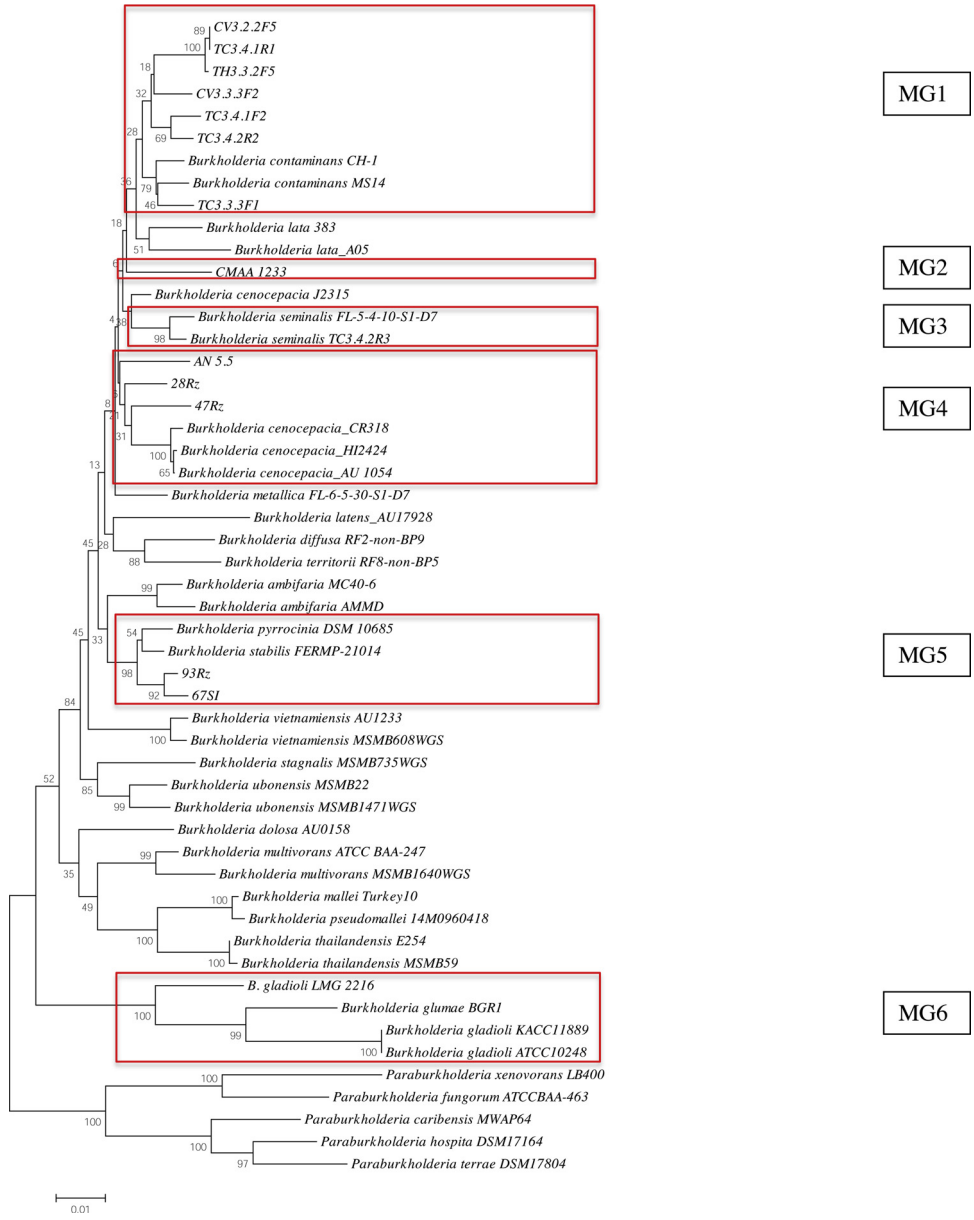


**Fig. 1.** Phylogenetic reconstruction based on Multilocus sequence analysis (MLSA) of the *Burkholderia* spp isolates. The figure displays the unrooted neighbor-joining tree (Jukes and Cantor, 1969) based on MLSA (*atpD*, *gltB*, *gyrB*) data. The numbers in the branches indicate bootstrap values calculated using 1000 replications. The scale bar indicates 0.01 substitutions per nucleotide position.

BLASTx.

### 2.9. Growth and lipid peroxidation of *B. seminalis* TC3.4.2R3 under experimental conditions

The strain *B. seminalis* TC3.4.2R3 was grown in 50 ml TSB glucose (10 %) under four distinct experimental conditions, as follows: TC3.4.2R3 alone (control), TC3.4.2R3 + paraquat (250 μM), and TC3.4.2R3 + Orchid exudates. Each individual flask was kept shaking (150 rpm) at 28 °C for 72 h. Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substance – TBARS (Heath and Packer, 1968; Monteiro et al., 2011) in the cells. Malondialdehyde (MDA) was quantified by measuring the absorbance at 535 and 600 nm, and the concentration was calculated using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

### 2.10. Total RNA isolation and cDNA synthesis

Bacteria cells were harvest by centrifugation (15 min at 6000 rpm) and the RNA was isolated using the PureLink® RNA Mini Kit (Ambion, Foster City, CA, USA). RNA samples were resuspended in 30 μl of water and stored at −80 °C. The integrity and quantity of extracted RNA were verified in a 1.2 % denaturing agarose gel prepared with MOPS 200 mM, sodium acetate 50 mM, EDTA 10 mM, formaldehyde (0.7 %) and SYBR safe gel stain. The RNA concentration was determined using NanoDrop ND-1000 (Thermo Scientific, USA). All materials used for RNA work were treated with DEPC to eliminated RNase. Total RNA (0.5 μg) was reverse-transcribed into cDNA using random hexamer primers (Invitrogen, Carlsbad, CA, USA) and 200 U Superscript III RNase $H^-$ reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's manual. For each RNA sample, a negative RT (no addition of reverse transcriptase) was performed and used as a negative control in subsequent PCRs.

**Table 3**
*In vitro* susceptibility of *Burkholderia* strains ($n$ = 15) tested for 9 antibiotics[a].

| Strain | MLSA group | PIP | CAR | TIC | ATM | IPM | SZT | DOX | TET | MIN |
|---|---|---|---|---|---|---|---|---|---|---|
| CV3.3.3F2 | MG1 | S[b] | I | I | S | S | S | I | S | I |
| TC3.3.3F1 | | R | R | R | S | S | S | S | I | S |
| TH3.3.2F5 | | R | R | R | I | I | S | I | R | S |
| CV3.2.2F5 | | I | R | R | R | S | S | S | R | S |
| TC3.4.2R2 | | R | R | R | S | S | S | S | S | S |
| TC3.4.1R1 | | I | R | R | S | S | S | S | I | S |
| TC3.4.1F2 | | R | R | R | I | R | S | S | I | S |
| CMAA 1233 | MG2 | R | R | R | R | I | S | S | R | S |
| TC3.4.2R3 | MG3 | R | R | R | S | S | S | S | S | S |
| AN 5.5 | MG4 | S | I | I | S | S | S | I | S | I |
| 28Rz | | S | I | I | S | S | S | S | S | S |
| 47Rz | | I | I | I | S | S | S | I | S | I |
| 67SI | MG5 | S | S | I | S | S | S | I | S | I |
| 93Rz | | R | R | R | R | S | R | R | R | S |
| LMG2216 | MG6 | S | S | I | S | S | S | I | S | S |

[a] Corresponding names are described in Table 2.
[b] S = sensitive, R = resistant, I = intermediate.

### 2.11. Expression of genes associated with oxidative stress

The primer design used to amplify the target genes in this study were carried out using *Primer 3* v. 0.4.0 (http://frodo.wi.mit.edu/), and the draft genome of *B. seminalis* TC3.4.2R3 as a reference (Araújo et al., 2016). First, conventional PCR was used to validate the design primers. All amplification products of each of the five genes (one *sod* gene and four *kat* genes) were purified, sequenced and compared to the GenBank data using BLASTn (http://blast.ncbi.nlm.nih.gov/) in order to confirm amplicon specificity.

The qPCRs were done on a StepOne Plus (Applied Biosystems, Foster City, CA, USA) thermocycler programmed to an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C. The specificity of qPCR primer sets was evaluated using melting curves with a gradient from 60 to 96 °C, ranging 1 °C each 30 s. Each amplification reaction was performed containing 2 µl of cDNA (ca. 16 ng), 10 µM of each primer and the Platinum SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). The DNA recombination gene *recN* was used as a reference gene. The StepOne Plus Software was used to determine the relative quantification of the target genes in comparison to the reference gene. Gene expression data were statistically compared using the Student *t*-test (*t*-test, $\alpha = 0.05$).

### 2.12. Statistical analysis

Statistical differences were tested using one-way analysis of variance (ANOVA), followed by Duncan test. The level of significance was set at $P < 0.05$ for all experiments. All statistical analyses were carried out using R software v. 2.15.1. For correlational analysis between MLSA profiles and antibiotic susceptibility data, and MLSA profiles and bacterial traits (i.e., synthesis of antimicrobial compounds, virulence against *G. mellonella*, and control of orchid necrosis), we used a non-parametric Mantel-type test implemented as the RELATE routine in PRIMER6+.

## 3. Results

### 3.1. Phylogenetic analysis of Burkholderia spp

Our collection of *Burkholderia* spp. strains were obtained from distinct environmental samples (e.g., soil, rhizosphere and roots), most of which arose from sugarcane plants (*Saccharum* spp.) (see Table 1 for details). Only 2 strains, i.e. TC3.4.2R3 and LMG2216, were previously identified as *B. seminalis* (Araújo et al., 2016) and *B. gladioli,* respectively, while the species of the others remain unassigned. To determine phylogenetic relationships and taxonomic characteristics of these strains, we used MLSA based on three genes (*atpD*, *gltB* and *gyrB*), which were previously validated for the analysis of the Bcc group (Baldwin et al. 2005). Except for *B. gladioli* LMG2216, all strains clustered into well-supported clades by parsimony analysis within the Bcc species (Fig. 1). The *B. seminalis* strain TC3.4.2R3 – a sugarcane endophytic isolate – clustered in a clade with *B. seminalis* FL-5-4-10-S1-D7 – an isolate obtained from soil. Strains CV3.2.2F5, TC3.4.1R1, TH3.3.2F5, CV3.3.3F2, TC3.4.1F2, TC3.4.2R2 and TC3.3.3F1 – all of which were obtained from sugarcane – clustered into a clade that includes *B. contaminans*, even though these isolates clustered together in a divergent group. The strain CMAA1233 is in a divergent clade with no similarity with any of the identified species used in this study, suggesting it is a possible new species within the Bcc group. The strains AN5.5, 28RZ and 47RZ clustered within a divergent clade related to *B. cenocepacia* strains CR318, HI2424 and AU1054, albeit this clade does not include the epidemic strain *B. cenocepacia* J2315. The strains 93RZ and 67SI grouped in a well-supported clade with *B. stabilis* and *B. pyrrocinia.* Overall, the MLSA analysis revealed a total of six groups (MG), as follows: MG1 (strains CV3.2.2F5, TC3.4.1R1, TH3.3.2F5, CV3.3.3F2, TC3.4.1F2, TC3.4.2R2 and TC3.3.3F1), MG2 (strain CMAA1233), MG3 (*B. seminalis* strain TC3.4.2R3), MG4 (strains AN5.5, 28RZ and 47RZ), MG5 (strains 93RZ and 67SI) and MG6 (*B. gladioli* LMG2216) (Fig. 1).

We also determined differences in growth rate across these isolates. The results revealed that all isolates had similar growth curves, reaching the stationary phase after ca. 12 h of incubation. The only exception was the *B. gladioli* LMG2216, which required 20 h of incubation to reach a stationary phase (Fig. S1).

### 3.2. Antimicrobial susceptibility and correlational analysis

The susceptibility of these strains to 16 antimicrobials belonging to nine different classes (Table 2) was examined using disk diffusion assays (Table 3). Overall, all tested strains were sensitive to cephalosporins, ceftazidime and cefepime, levofloxacin (fluoroquinolone), meropenem (carbapenem), tigecycline (glycylcycline), piperacillin + tazobactam (β-lactam), and chloramphenicol. Most of the strains belonging to the MLSA MG1 group were resistant to β-lactams (Piperacillin, Carbenicillin and Ticarcillin + Clavulanate), albeit the strain CV3.3.3F2 was sensitive/intermediate. In the MG5 group, the strain 67SI was sensitive/intermediate to all antibiotics, while 93RZ was resistant to Sulfamethoxazole-Trimethoprim, Piperacillin, Ticarcillin + Clavulanate, Carbenicillin, Aztreonam, Doxycycline and Tetracycline (Table 3). In addition, the strains CV3.3.3F5 and TH3.3.2F5 (MG1), CMA1233 (MG2), and 93RZ (MG4) were resistant to tetracycline. Last, only the strains TC3.4.1F2 and 93RZ were resistant to imipenem and doxycycline, respectively (Table 3).

The strain TC3.4.2R3 (MG3) was resistant to the β-lactams, such as Piperacillin, Carbenicillin and Ticarcillin + Clavulanate, but was sensitive to Aztreonam, Imipenem, Sulfamethoxazole-Trimethoprim, Doxycycline, Tetracycline, Minocycline. We further investigated the genome of this strain TC3.4.2R3 (GenBank Accession number LAEU00000000) and successfully identified genes encoding 5 antibiotic efflux pumps and 2 genes (Bsem_05019 and Bsem_05476) encoding β-lactamase enzyme, which is known to be associated with resistance to β-lactams and cephalosporin (Table S1).

We performed correlational analysis between the MLSA clustering profile and the antibiotic susceptibility data across all 15 *Burkholderia* strains. The result of a pairwise correlation between genetic (MLSA) clustering and sensibility to antibiotics showed that these traits are not significantly correlated (rho = 0.05; $P = 0.32$). We also used genomic information of the strain TC3.4.2R3 to identify genes potentially associated with antibiotic resistance that could be acquired by horizontal gene transfer. By combining BLASTx against CARD, we identified 76 genes associated with potential antibiotic resistance, of which 25 were in chromosome 1 (CR1), 46 in chromosome 2 (CR2), and 5 in the

**Table 4**
Virulence to *Galleria mellonella,* control of orchid necrosis, and antimicrobial activity of *Burkholderia* spp. The synthesis of antimicrobial compounds was evaluated *in vitro* by the presence of inhibition halos.

| Strain | MLSA group | Virulence to *G. mellonella*[a] | Control of orchid necrosis[b] | Eco[c] | Bsp[c] | Cep[d] | Fuv[d] | Asf[d] |
|---|---|---|---|---|---|---|---|---|
| CV3.3.3F2 | MG1 | 1 | + | I[e] | I | I | I | I |
| TC3.3.3F1 | | 1 | + | I | I | I | I | I |
| TH3.3.2F5 | | 2 | + | I | I | I | I | I |
| CV3.2.2F5 | | 2 | + | I | I | I | NI | I |
| TC3.4.2R2 | | 1 | + | I | I | I | I | I |
| TC3.4.1R1 | | 1 | – | I | I | I | I | I |
| TC3.4.1F2 | | 1 | – | I | I | I | I | I |
| CMAA 1233 | MG2 | 3 | + | NI | I | NI | I | NI |
| TC3.4.2R3 | MG3 | 2 | + | I | I | I | I | NI |
| AN 5.5 | MG4 | 3 | + | NI | I | I | NI | I |
| 28Rz | | 2 | – | NI | I | I | I | I |
| 47Rz | | 1 | + | NI | I | I | I | I |
| 67SI | MG5 | 2 | – | I | NI | I | I | I |
| 93Rz | | 2 | + | I | NI | I | I | I |
| LMG2216 | MG6 | 3 | NR | NI | I | I | I | I |

[a] Mortality at 7th days: 1 = > 95 %; 2 = range from 60 to 85 %; 3 = < .25 %.
[b] +: control of orchid necrosis caused by *B. gladioli;* -: no control of orchid necrosis; NR: not tested.
[c] *Escherichia coli (*Eco) and *Bacillus* sp (Bsp).
[d] *Ceratocystis paradoxa* (Cep), *Fusarium verticillioides* (Fuv) and *Aspergillus fumigatus* (Asf).
[e] I: presence of inhibition halo and NI: absence of inhibition halo.

plasmid (P3) (Table S2). From these, two of the genes found in CR1, in addition to three genes in CR2, and two in P3; were found within genomic islands in the genome. Most interestingly, some of them were associated with protein transport and efflux pumps (see Table S2 in Supporting Material for details).

### 3.3. Synthesis of antimicrobial compounds, virulence against G. mellonella, and potential control of orchid necrosis

The antibacterial and antifungal activities of *Burkholderia* spp. strains were tested on PDA (fungi) and TSA (bacteria) plates after 5 or 2 days, respectively, against several bacterial and fungal species. Overall, the 15 strains successfully inhibited the Gram-positive bacterium *Staphylococcus aureus* and the phytopathogenic fungi *Colletotrichum* sp. and *Ceratocystis fimbriata* (Table 4) while none inhibited the multidrug-resistant bacterium *Pseudomonas aeruginosa.*

The strains within the MG1 displayed a wider inhibitory spectrum than strains from other MLSA MGs, including virulence against *G. mellonella* and the ability to control orchid necrosis. For example, all strains inhibited the bacteria *Escherichia coli, Bacillus* sp., and the fungi *Ceratocystis paradoxa* and *Aspergillus fumigatus,* and only the strain CV3.2.2F5 was not able to inhibit the fungus *Fusarium verticillioides.* In addition, all strains caused greater than 60 % mortality of *G. mellonella*, and five (out of seven) strains caused 100 % of larvae mortality. Five strains within the MG1 were also able to control orchid necrosis (Table 4). In general, strains from MLSA groups MG2, MG3, MG4, MG5 and MG6 were less virulent against *G. mellonella* and in the evaluated conditions, the 5 strains from the groups MG2, MG4 and MG6 were not able to inhibit *E. coli.* The two strains from MG4 (i.e. 67SI and 93Rz) did not inhibit *Bacillus* sp. but inhibited all tested fungi. Last, we found a significant correlation between MLSA clustering profile and the collection of tested traits (i.e., synthesis of antimicrobial compounds, virulence against *G. mellonella*, and control of orchid necrosis) (rho = 0.51; $P = 0.004$).

### 3.4. Plant beneficial activities

Strains used in the present study were originally isolated from soil, rhizosphere or inside plants tissues (endophytes) and except for the strain LMG 2216, which was identified as *B. gladioli*, (a known plant pathogen), all others had no deleterious effect on plant growth. Cellulases and siderophore production and phosphate solubilization were observed for all evaluated strains. In addition, as reported before, 10 strains inhibited orchid necrosis caused by *B. gladioli* (Table 4).

### 3.5. B. seminalis TC3.4.2R3 as a model organism for risk assessment and biotechnological application

We further explored the strain TC3.4.2R3 as a model organism for *B. gladioli* control. Inoculation of *B. gladioli* in orchid leaves induced necrosis (Fig. 2a), while the co-inoculation with *B. seminalis* TC3.4.2R3 suppressed these symptoms (Fig. 2b). This result confirmed the activity of the endophyte *B. seminalis* as a biological control agent against the phytopathogen *B. gladioli.* Plants inoculated with PBS (negative control) and *B. seminalis* TC3.4.2R3 did not display any symptoms of orchid necrosis (Fig. 2c,d). Of key relevance, we also found that the disease was not suppressed when the endophytic bacterium was inoculated 1 cm apart from the *B. gladioli* inoculation point. Moreover, the inoculation of *B. seminalis* in abaxial leaf tissues by infiltration 6 days prior to *B. gladioli* suppressed orchid necrosis. However, the suppression of the disease was observed only in the zone that both endophytic and pathogenic bacteria were in contact (Fig. 2e). Collectively, these results indicate that the plant colonization by *B. seminalis* is necessary to

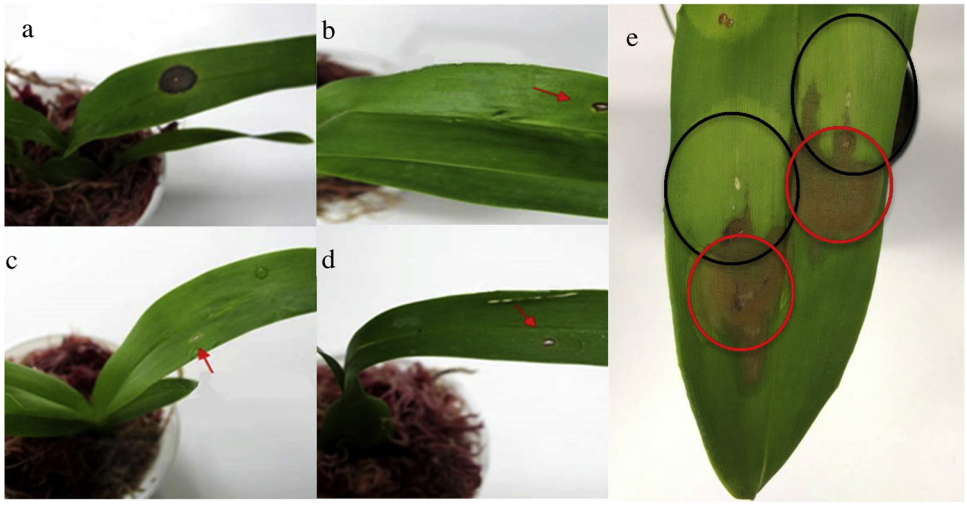


**Fig. 2.** Control of orchid necrosis caused by *Burkholderia gladioli* in leaf fragments of *Oncidium flexuosum* 'Aloha Iwanaga'. (a) symptoms caused by the inoculation of *B. gladioli;* (b) suppression of the symptoms due to the co-inoculation of *B. gladioli* and *B. seminalis* TC3.4.2R3; (c) leaf inoculated with *B. seminalis* TC3.4.2R3; (d) negative control, i.e. leaf inoculated with PBS buffer, and (d) inoculation of *B. gladioli* (red circle) 2 cm apart from *B. seminalis* TC3.4.2R3 (black circle). All photographs were taken 5 days after inoculation.

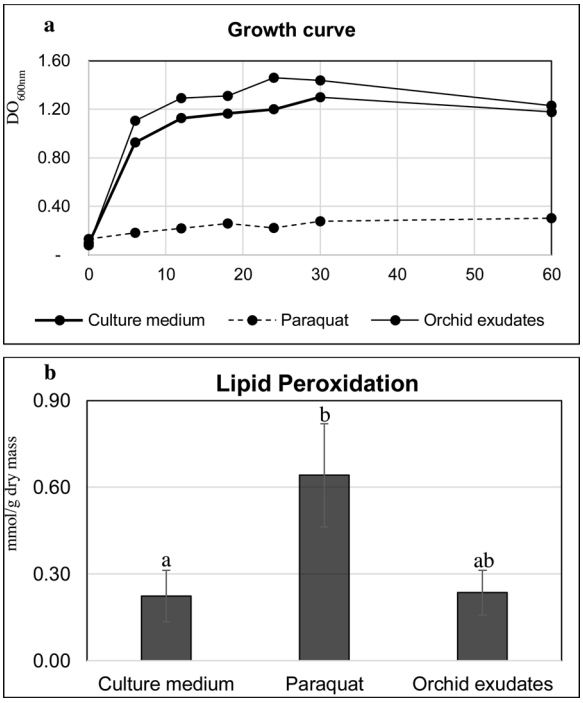


**Fig. 3.** Bacterial growth and lipid peroxidation. (a) *B. seminalis* TC3.4.2R3 growth curve in culture medium (10 % TSB), culture medium supplemented with Paraquat (250 μM) or orchid root exudates. (b) Lipid peroxidation of the bacterial membrane after exposure to culture medium (10 % TSB), culture medium with Paraquat (250 μM) or orchid root exudates.

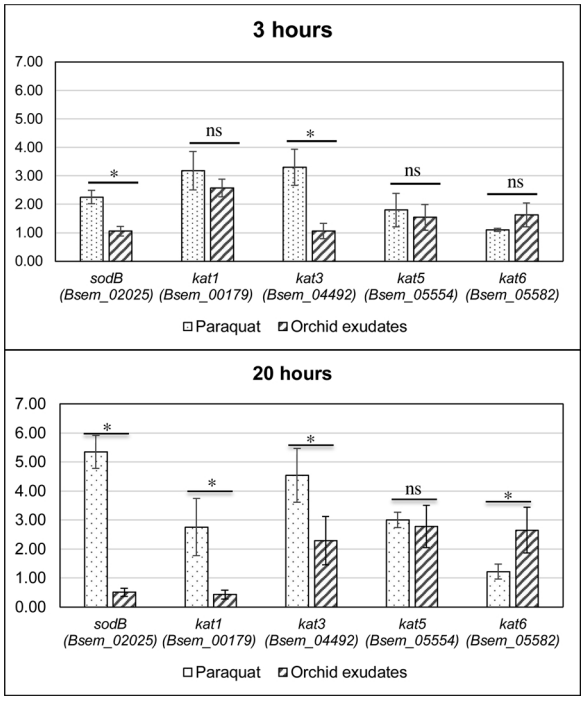


**Fig. 4.** Relative of expression of *sodB, kat1, kat3, kat5* and *kat6* genes *B. seminalis* TC3.4.2R3 exposed to paraquat (250 μM) and orchid root exudates. The expression profiles were evaluated at 3 h after bacteria inoculation (top panel) and (b) 20 h after bacteria inoculation (bottom panel). The *recN* (DNA repair protein) was used as a reference gene. The results are shown as the average of three replicates and * indicates statistically significant differences ($\alpha = 0.05$) based on Student *t*-tests.

guarantee physical contact between the endophyte and the phytopathogen, thus promoting orchid necrosis suppression. Previous results have shown that this strain TC3.4.2R3 is not able to induce orchid systemic resistance (data not shown).

We also investigated the bacterial responses to orchid exudates. Since plant defense may induce oxidative stress in plant-associated bacteria, we also compared the bacterial response to the herbicide paraquat. Overall, these results revealed that whereas the plant exudates stimulated bacterial growth, the presence of paraquat was inhibitory to bacterial growth (Fig. 3A), in addition, paraquat was found to induced peroxidation (Fig. 3B).

The expression of five *B. seminalis* TC3.4.2R3 genes related to antioxidative responses (*sodB, kat1, kat3, kat5* and *kat6*) was evaluated after 3 h and 20 h of exposure to oxidative stress induced by paraquat and orchid root exudates. Overall, the expression of these oxidative stress-associated genes was regulated by orchid root exudates and paraquat in a specific manner. The *sodB* gene expression was only induced by paraquat, while *kat6* was consistently induced by root exudates. At 20 h, the expression of *sodB* and *kat1* was induced by paraquat, but repressed by orchid root exudates, indicating that these genes are likely specific for the protection against oxidative stress induced by paraquat. The *kat3* gene was only induced in the presence of paraquat at 3 h, but by both paraquat and orchid root exudates at 20 h (Fig. 4). The *kat5* gene expression was induced by both conditions at 3 and 20 h (Fig. 4), thus suggesting a potential role in general oxidative stress response of this bacterium.

## 4. Discussion

*Burkholderia* species are often isolated from the endosphere, phyllosphere and rhizosphere of many plant species, or isolated from chronic infection in immunocompromised patients, especially chronic cystic fibrosis patients (Eberl and Vandamme, 2016). These isolates can be genetically distinct but phenotypically similar and is not possible to discriminate strains from soil, plant or patients. These bacteria present high phenotypic plasticity, determined by differential response to environmental stimulus, which is likely an important, yet unexplored mechanism of *Burkholderia* members to occupy different niches and survive in distinct and often contrasting environments, such as the human lung and the plant rhizosphere.

The adaptive mechanisms that allow these bacteria to colonize different host are still uncertain, but Nunvar et al. (2017) observed that the host immune system can modulate *B. cenocepacia* evolution during chronic CF infection since genes that encode proteins involved in the protection against hydrogen peroxide and hypochlorous acid are more frequently mutated in sputum isolates of CF-patients. This indicates that mutation, but not gene transfer, in these genes is likely associated with the bacterial persistence in the airways.

In this study, we compared 15 *Burkholderia* spp. isolates obtained from distinct environmental samples to evaluate the association between taxonomic profile and traits associated to virulence to immunocompromised patients (antibiotic resistance and virulence to the model *G. mellonella*) and colonization of the rhizosphere and the host plant. Remarkably, 14 (out of 15) strains belonged to the Bcc, confirming previous observations that members of the Bcc group, while potentially pathogenic for immunocompromised people, are highly prevalent as plant-associated organisms.

Based on MLSA, these isolates were clustered into 6 groups (MG01 to MG06). The group MG04 clustered with strains CR318, AU1054 and HI2424. A recent study (Wallner et al., 2019) had proposed separate *B. cenocepacia* species in human pathogenic and plant-adapted species. *B. cenocepacia sensu strict* includes the epidemic strain J2315, and a second species commonly found in association with plants, named *B. servocepacia* (including the strains CR318, AU1054 and HI2424). The authors observed that the plant-adapted strains carry genes for the utilization of plant derivatives and are often competing and surviving in soil. On the

other hand, the *B. cenocepacia* carry genes associated with virulence and are survival in humans. In fact, the members of MG04 (strains AN 5.5, 28Rz and 47 Rz) were sensitive/intermediate to all antibiotic used to compare the strains (Table 3) and inhibit most of the microorganisms evaluated, except *E. coli* that is associated to humans (Table 4). These findings provide evidence that these these bacteria are more adapted to soil-plant systems than to animal hosts.

The clustering based on MLSA analysis showed no correlation with antibiotic sensitivity, thus suggesting that at least some of the antibiotic resistance genes could be potentially acquired by horizontal gene transfer. While 9 out of 76 (i.e., 11.8 %) antibiotic resistance-associated genes were present in clearly define genomic islands, we could not rule out that other genes might be present in horizontally acquired sequences in which the classical features of genomic islands had been lost for this strain.

In contrast, the ability to produce antimicrobial and antifungal compounds was more prevalent in the MG1 MLSA group. Indeed, these isolates showed a significantly wider inhibitory spectrum than strains from other MLSA groups, including the capacity to control orchid necrosis. Collectively, these results showed that there is a correlation between the production of antifungal and antibacterial compounds and the MLSA clusters, suggesting that most of the genes that control these traits are acquired by vertical gene transfer. The ability to produce antimicrobial compounds by *Burkholderia* spp. is an important feature promoting niche adaptation, since these bacteria must compete in the soil and rhizosphere, before the plant colonization. Using this ability to inhibit other microorganisms, certain strains are known to suppress plant diseases. For example, *B. seminalis* strain R456, which was isolated from the rhizosphere of rice, was shown to reduce the incidence and severity of rice sheath blight under greenhouse conditions (Li et al., 2011), *B. seminalis* TC3.4.2R3 was isolated from sugarcane roots, but shown to be effective in the control of the orchid necrosis (Araújo et al., 2016), while *B. cenocepacia* CACua-24, isolated from sugarcane rhizosphere, produced antifungal and antibacterial compounds against a broad range phytopathogens. In our study, most of the *Burkholderia* strains were able to control orchid necrosis, caused by *B. gladioli,* and to produced antimicrobial compounds against *Escherichia coli, Bacillus* sp., *Ceratocystis paradoxa* and *Fusarium verticillioides.*

Based on these traits, we selected *B. seminalis* TC3.4.2R3 to further evaluate interactive aspects with orchid plants, especially under oxidative stress conditions. This is of key relevance since the ability to cope with oxidative stress is directly associated with the skill of distinct strains to colonize and thrive across distinct gradients of environmental conditions, including plant and patients. Niche adaptation depends on the capability of organisms to explore and exploit nutrients and to cope with local environmental conditions. In the present study, the orchid root exudate, despite its potential to induce oxidative stress, promoted a significant growth of *B. seminalis* TC3.4.2R3 when compared to the standard culture medium. In contrast, the bona fide pro-oxidant paraquat significantly reduced this bacterium growth (Fig. 3A). The presence of paraquat (but not root exudates) was found to increased membrane lipid-mediated peroxidation. Collectively, these findings suggest that paraquat triggered ROS production leading to membrane damage, and thus inhibition of the bacterial growth. In contrast, root exudates, which are generally composed of sugars (e.g., galactose, mannitol, fructose, arabitol, dulcitol and ribitol) and organic acids (LeFevre et al., 2013; Li et al., 2013), were not found to significantly induce membrane damage, but induced the bacterial growth.

Moreover, plant exudates were found to trigger the bacterial antioxidant response. For instance, in *B. glumae* superoxide dismutase (*sod*) and catalase (*kat*) genes are upregulated after 30 h, thus likely playing an important role in pathogenicity and protection against visible light (Chun et al., 2009). Similarly, in *Sinorhizobium meliloti, katA* and *sodC* genes were induced during the interaction with *Medicago* plants (Ampe et al., 2003), suggesting the role of these genes in the bacterium colonization of the host plant. Likewise, the virulence of *Burkholderia* spp. in macrophages is related to oxidative responses (Keith and Valvano, 2007; Vanaporn et al., 2011). Therefore, the ability to cope with oxidative stress is an important strategy of *Burkholderia* species used to colonize different hosts, such as plant and immunocompromised patients. Our findings showed that *B. seminalis* TC3.4.2R3 responded to oxidative stress differentially when challenged by paraquat and root exudate, indicating that this bacterium uses a different strategy for oxidative stress protection provided from diverse sources, which could explain the phenotypic plasticity that allow these bacteria to colonize a broad range of taxonomically different hosts.

The adaptation of *B. seminalis* to specific environments is highly associated with its unique metabolic capacity, which according to Zhu et al. (2016) may be strain-specific and generally linked with genomic variants and niche-dependent differential expression of the corresponding genes. Regulating the expression of different genes in response to these environments, *Burkholderia* spp. could present the plasticity that allow them to occupy different niches. This plasticity strategy can promote increasing fitness, and, once coupled with mutation and selection, can result in a wide range of adaptation across contrasting environmental conditions (Nunvar et al., 2017).

## 5. Conclusion

In conclusion, we found evidence supporting the ability of *Burkholderia* spp. to establish and to persist into immunocompromised patients, plants and soil are likely associated with genetic variation. However, the evolution of these traits seems to occur in different ways. Traits associated with antibiotic resistance appear to be more likely strain-specific, with potentially no correlation with taxonomic variations. This lack of correlation could be due to the selection of adaptative mutations during chronic infection that increase the ability of some strains to persist in the host. On the other hand, traits associated to soil and plant colonization correlate with the taxonomic profile, being likely acquired by vertical gene transfer. Collectively, our results indicate that selection of specific strains can be used for plant protection with low risk for immunocompromised patients. We advocate further studies are needed to broaden the spectrum of isolates and environmental samples to corroborate our findings, thus providing knowledge on the ecological niche adaptation and evolution of *Burkholderia* spp. across disparate systems.

## Author contributions statement

**Welington Luiz Araújo:** funding acquisition, project administration, designed the study, supervision and write the manuscript.

**Miguel A. Valvano and Welington Luiz Araújo:** designed the study, discussed the results and write the manuscript.

**Karent** J. **Romero-Gutiérrez:** performed the phenotypic characterization of *Burkholderia* strains.

**Manuella N. Dourado:** performed the interaction between *Burkholderia* strains and orchid plants.

**Leandro M. Garrido and Luiz Ricardo Olchanheski:** performed genomic analysis.

**Francisco Dini-Andreote:** performed the statistical analysis and discussed the results.

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## Declaration of Competing Interest

The authors declare that there is no conflict of interest.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126451.

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