Plant Disease • 2024 • 108:2376-2388 • https://doi.org/10.1094/PDIS-09-23-1823-RE

Genetic Diversity and Virulence of Phytopathogenic *Burkholderia glumae* Strains Isolated from Rice Cultivars in Valleys of the High Jungle of Perú

Renzo A. Valdez-Nuñez,[1,2,†] Lucero C. Ramos-Luna,[2] Patricia P. Meza-Catalán,[2] Nayza R. Asencios-Sifuentes,[2] Angel W. Ocaña-Rodriguez,[1] Julio C. Chávez-Galarza,[1] Ana N. Sandoval-Vergara,[3] and Gilles Béna[4]

[1] Departamento Académico de Ciencias Básicas, Facultad de Ingeniería, Universidad Nacional de Barranca, Barranca 15169, Lima, Perú
[2] Biotechnology Research Laboratory, Universidad Nacional de Barranca, Barranca 15169, Lima, Perú
[3] Departamento Académico Agrosilvopastoril, Universidad Nacional de San Martín, Tarapoto, Perú
[4] IRD, CIRAD, INRAe, Institut Agro, PHIM (Plant Health Institute of Montpellier), University of Montpellier, Montpellier, France

Abstract

Burkholderia glumae causes bacterial leaf blight in rice, and its global spread has been exacerbated by climate change. To understand the genetic diversity and virulence of *B. glumae* strains isolated from rice cultivars in Perú, 47 isolates were obtained from infected rice fields, all belonging to *B. glumae*, and confirmed by *rec*A and *tox*B sequences. The BOX-PCR typing group has 38 genomic profiles, and these turn into seven variable number tandem repeats (VNTR) haplotypes. There was no correlation between clustering and geographical origin. Nineteen strains were selected for phenotypic characterization and virulence, using both the maceration level of the onion bulb proxy and inoculation of seeds of two rice cultivars. Several strains produced pigments other than toxoflavin, which correlated with onion bulb maceration. In terms of virulence at the seed level, all strains produced inhibition at the root and coleoptile level, but the severity of symptoms varied significantly between strains, revealing significant differences in pathogenicity. There is no correlation between maceration and virulence scores, probably reflecting different virulence mechanisms depending on the host infection stage. This is the first study to evaluate the VNTR diversity and virulence of Peruvian strains of *B. glumae* in two commercial cultivars.

Keywords: *Burkholderia glumae*, pathogen diversity, rice, toxoflavin, virulence assays, VNTR

Burkholderia sensu lato (s.l.) (Yabuuchi et al. 1992) are a group of Gram-negative bacteria that are ubiquitous in various ecological niches and belong to the subphylum β-proteobacteria. Currently, through phylogenetic analyzes of conserved genes and comparative genomics, the *Burkholderia* s.l. has been split into seven new genera: *Burkholderia* sensu stricto (s.s.), *Caballeronia* (Dobritsa and Samadpour 2016), *Paraburkholderia* (Sawana et al. 2014), *Robbsia* (Lopes-Santos et al. 2017), *Mycetohabitans*, *Trinickia* (Estrada-de Los Santos et al. 2018), and *Pararobbsia* (Lin et al. 2020). The *Burkholderia* genus includes the famous *B. cepacia* complex (Bcc), composed of at least 20 different species that are known to be either human pathogenic or opportunistic bacteria. The *Burkholderia* genus is made up of species from diverse ecological niches with a large metabolic versatility (Jin et al. 2020). Among them, three are notably phytopathogenic species, i.e., *B. glumae* (Ham et al. 2011), *B. gladioli* (Fonseca-Guerra et al. 2022), and *B. plantarii* (Wang et al. 2016).

This association between plants and microorganisms is carried out at the level of the rhizosphere, endosphere, and/or phyllosphere (Afzal et al. 2019). The relationships between plants and phytopathogenic *Burkholderia* species constitute a phytosanitary problem worldwide (Ham et al. 2011; Ortega and Rojas 2021). *B. glumae*, *B. gladioli*, and *B. plantarii* are the causative agents of bacterial rice panicle blight in several countries in Southeast Asia (Jungkhun et al. 2022; Ramachandran et al. 2021), North America (Lee et al. 2021), and Latin America (Béna et al. 2021; Riera-Ruiz et al. 2014; Valdez-Nuñez et al. 2020). *B. glumae* can infect the crop at any stage, causing rot and delay in seed growth until grain vanishing during the reproductive stage (Ortega and Rojas 2021). It is postulated that high humidity and high night temperatures during the flowering stage (Zhou 2019), accompanied by nutritional factors, such as excessive doses of nitrogen fertilizers, exacerbate the severity of the infection (Kim et al. 2018). Several virulence factors are involved in the development of the disease, among which is the production of toxoflavin. Toxoflavin is a yellow pigment diffusible in the medium with antimicrobial and phytotoxic activity, which acts as the main virulence factor specific to *B. glumae* and *B. gladioli* (Ham et al. 2011; Ortega and Rojas 2021), although recent reports confirm its biosynthesis by *Pseudomonas alcaliphila* (Faucher et al. 2022). Type 3 and 6 secretion systems, production of lipases, and polygalacturonases are also involved in the bacterial pathogenic activity (Mannaa et al. 2019).

In northern Perú, Valdez-Nuñez et al. (2020) reported the presence of *B. glumae* and *B. gladioli* as causes of bacterial panicle blight, revealing a high genetic diversity (nine BOX-PCR groups), numerous virulence factors, and resistance to oxolinic acid and H_2O_2 in relation to the geographical areas of origin. Rice cultivation is one of the main agricultural activities in the San Martín region in Perú. However, bacterial panicle blight, potentially exacerbated by current climate change, constitutes a threat to this production. Due to the above, developing new strategies to control bacterial panicle blight is urgent, which must be addressed first through the study of genetic, phenotypic, and virulence diversity of phytopathogenic *Burkholderia* isolates recovered from rice fields in this region. The objectives of this work were to (i) isolate grain endophytic bacteria (putative phytopathogens) from rice panicles with disease symptoms in four valleys of the San Martín region, (ii) identify phytopathogenic *Burkholderia* strains with specific molecular markers, (iii) study the genetic diversity of *B. glumae* strains through microsatellite sequencing, and (iv) characterize the virulence of these strains.

Materials and Methods

Collection of rice samples

Grain samples of various cultivars of rice from the Bajo Mayo, Altomayo, Alto Huallaga, and central Huallaga valleys in the San

[†]Corresponding author: R. A. Valdez-Nuñez; rvaldez@unab.edu.pe

Funding: This work was supported by the Fondo Nacional de Desarrollo Científico, Tecnológico y de Innovación Tecnológica (FONDECYT) grant number CONTRATO N°062-2021-FONDECYT.

The author(s) declare no conflict of interest.

Accepted for publication 19 February 2024.

Martín region were collected (Fig. 1). The grains from panicles with symptoms of bacterial blight were collected according to Nandakumar et al. (2009). The panicles were stored in paper bags at room temperature. Georeferencing data and the cultivation history of each of the samples were taken and are given in Table 1.

Isolation of endophytic bacteria from grains of rice

Phytopathogenic endophytic bacteria were isolated from grains of panicles with symptoms of bacterial blight. The grains were disinfected on surface according to Nandakumar et al. (2009) and streaked in a modified CCNT selective medium (Kawaradani et al. 2000) containing 2.0 g of yeast extract; 1.0 g of peptone; 4.0 g of meso-inositol; 0.01 g of cetrimide; 0.01 g of chloramphenicol; 0.01 g of novobiocin; and 18.0 g of agar in 1 liter of distilled water that was adjusted to a pH of 4.8. The rice grains were placed on the surface of the plates and incubated at 41°C for 4 days. The isolates obtained were purified by the streaking technique in Petri dishes containing Tryptic Soy Agar (TSA)

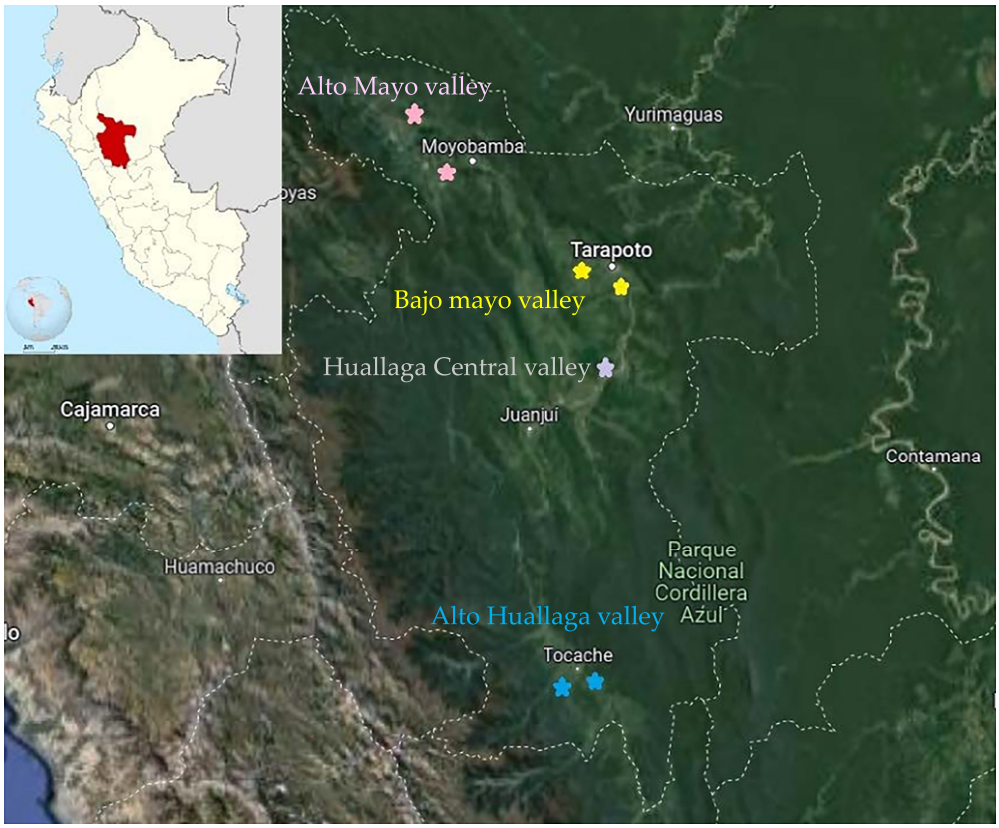

Fig. 1. Geographical locations of the rice valleys where the grains were collected.

Table 1. Geographical location of rice collections and distribution of phytopathogenic *Burkholderia* strains isolated from rice cultivars from rice valleys in the San Martin region

Valley	Province	District/sector	Rice cultivar	Geographic coordinates	Number of samples	Number and code of *Burkholderia* strain
Alto Mayo	Rioja	Awajun/San Francisco de Amayo	Valor, La Esperanza, and La Victoria	5°46′43″S, 77°18′29″W; 916 meters above sea level (m.a.s.l.)	36	–
	Moyobamba	Moyobamba/La Conquista	Valor	5°52′41″S, 77°10′10″W; 913 m.a.s.l.	21	
Bajo Mayo	Lamas	Cacatachi/ Rosanaico-(A)	Valor and La Conquista	6°28′11″S, 76°26′26″W; 299 m.a.s.l.	15	(5)-F1/F2/F5/F21/F22
	San Martín	Juan Guerra/ Estación El Porvenir-(B)	La Conquista, Valor, Bellavista, La Esperanza, and Fedearroz 60	6°35′49″S, 76°19′32″W; 309 m.a.s.l.	15	(12)-F7/F8a/F9/F10/F11a/ F11b/F13a/F13b/F14a/F15/ F16/F19
Alto Huallaga	Tocache	Uchiza/Nueva Esperanza-(C)	Ferón, Valor, La Esperanza, and Pacamur	8°15′39″S, 76°33′59″W; 495 m.a.s.l.	15	(17)-F46a/F47/F49/F50b/F51/ F52a/F56/F58/F60b/F61/ F62/F63/F66/F67a/F69b/ F70/F71.
		Tocache/San Agustín de Huaquiche-(D)	Ferón and Valor	8°10′46″S, 76°30′09″W; 564 m.a.s.l.	15	(8)-F34/F35/F36/F40/F41/ F42/F43/F44
Huallaga Central	Picota	Picota/Santa Rocillo-(E)	Valor	6°55′32″S, 76°22′23″W; 218 m.a.s.l.	15	(5)-F72/F73/F74/F75b/F76

(Granucult, Millipore, Sigma Aldrich) until pure cultures were obtained.

Characterization and preservation of the collection

Isolates from grains were characterized according to their reaction to Gram staining (Somasegaran and Hoben 1994); morphocolonial and microscopic characteristics such as configuration, margin, elevation, color, and ease of removing colonies from the medium (Brown and Smith 2014); and toxoflavin production (Nandakumar et al. 2009). Toxoflavin production was assayed in King B medium (King et al. 1954) containing 20.0 g of peptone, 1.5 g of K_2HPO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, 15 ml of glycerol, and 15.0 g of agar-agar in 1 liter of distilled water that was adjusted to a pH of 7.2. Endophytic bacteria were streaked in Petri dishes containing King B agar and incubated at 37°C for 48 h. The production of diffused toxoflavin in the culture medium was evaluated according to the absence (−) or presence (+) of a fluorescent yellow pigment diffused in the culture medium. In both cases, these characteristics allowed probable phytopathogenic strains of *Burkholderia* to be identified. The preservation of the collection was carried out by cryopreservation at −20 and −80°C according to Garcia and Cotter (2016) and Cui et al. (2021).

Genomic DNA extraction

Genomic DNA was extracted from bacterial cultures incubated overnight in King B broth (25 ml) at 28°C, and after shaking at 150 rpm, the cell pellet was harvested by centrifugation at 13,000 rpm for 3 min (Bach et al. 2017). The commercial GenElute Bacterial Genomic DNA kit (Sigma Aldrich, U.S.A.) was used following the manufacturer's instructions. The DNA concentration was measured in a NanoDrop one spectrophotometer (Thermo Scientific, U.S.A.), and the quality was verified in 1% agarose gel using Diamond Nucleic Acid Dye (Promega, U.S.A.) and visualized in an electrophoresis chamber with black light (Cleaver Scientific Ltd., U.K.). The original DNA was stored at −20 and −80°C.

Molecular discrimination of phytopathogenic *Burkholderia*

The selection of strains belonging to the genus *Burkholderia* was carried out using specific primers from Spilker et al. (2009). We used the primers *rec*A-F (forward)5′-AGGACGATTCATGGAAGA WAGC-3′ and *rec*A-R (reverse)5′-GACGCACYGAYGMRTAG AACTT-3′ that were designed to amplify a 704-bp sequence of the *rec*A gene in *Burkholderia* species. The polymerase chain reaction (PCR) was performed in a Biometra thermal cycler (Analytic Jena, Germany). PCR reactions were performed using a 25-µl reaction mix containing 1.0 µl of template DNA (50 ng), 12.5 µl of KAPA Taq ReadyMix (2×) (Sigma-Aldrich, U.S.A.) (DNA polymerase, 0.05 units μl^{-1}, 3 mM $MgCl_2$, 400 µM each dNTP), 1.0 µl of each primer (5 pmol each forward and reverse), and 9.5 µl of PCR-grade water (Sigma-Aldrich, U.S.A.). The PCR was developed using an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, an extension for 60 s at 72°C, and a final extension of 5 min at 72°C. To visualize the amplicons, 2 µl of each PCR product were visualized on a 1.0% agarose gel in 0.5× TAE buffer according to Mahenthiralingam et al. (2000). In a second step, we proceeded to amplify the *tox*B gene (508 bp) that is part of the toxoflavin operon and encodes GTP cyclohydrolase II. The primers *tox*B_F: 5′-GCATTTGAAACCGAGATGGT-3′ and *tox*B_Rd: 5′-TCGCAT GCAGATAACCRAAG-3′, were used according to Bangratz et al. (2020). The PCR reaction mix was described above using the following PCR conditions: initial denaturation at 95°C for 12 min, followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, an extension for 45 s at 72°C, and a final extension of 7 min at 72°C. To visualize amplification, 2 µl of each PCR product were visualized on a 2.0% agarose gel in 0.5× TAE buffer. The positive amplification of the *tox*B gene allowed discriminating strains capable of producing toxoflavin. So far *tox*B has only been detected in *B. glumae* and *B. gladioli* and recently *P. alcaliphila*. All PCR products were purified and sequenced by MACROGEN Inc. (South Korea).

BOX-PCR genomic profiles

Genomic profiles were obtained by amplifying the genomic DNA of the *B. glumae* strains (selected after *rec*A and *tox*B sequences) using the BOX-A1R primer (5′-CTACGGCAAGGCGACGCTG ACG-3′) (Koeuth et al. 1995) in a Biometra Tone thermal cycler (Analytic Jena) following the cycle program described by Arone et al. (2014). PCR products were separated on a 1.5% agarose gel running at 60 V for 8 h and visualized in a black light electrophoresis chamber (Cleaver Scientific Ltd., U.K.). The DirectLoad 1 Kb DNA Ladder (Sigma Aldrich, U.S.A.) was used as a molecular weight marker. The genomic profiles of the generated individual strains were photographed, digitalized, and converted into a binary matrix of presence or absence of DNA bands. The cluster analysis allowed the construction of dendrograms using the free program DendroUPGMA (http://genomes.urv.es/UPGMA/) (Garcia-Vallvé et al. 1999) and applying the unweighted pair-group method with arithmetic mean (UPGMA) algorithm (Sneath and Sokal 1973) and the Jaccard t coefficient (Jaccard 1912), with 2% tolerance.

Variable number tandem repeats (VNTR) diversity in *B. glumae*

To estimate the within-species diversity of the *B. glumae* strains, one VNTR locus was amplified and sequenced using specific primers according to Béna et al. (2021). We used the primers BG12F (forward) (5′-CGGCCGATGTCCTTAAAC-3′)/BG12R (reverse) (5′-TAGTATT GACCGGCGTGAAA-3′) that encode an intergenic sequence located in chromosome 2 of the genome sequence of *B. glumae* $LMG2196^T$ (GCA_000960995.1). The PCR was performed in a Biometra thermal cycler (Analytic Jena, Germany). PCR reactions were performed using a 25-µl reaction mix containing 1.0 µl of template DNA (5 ng/µl), 12.5 µl of KAPA Taq ReadyMix (2×) (Sigma-Aldrich, U.S.A.) (DNA polymerase, 0.05 units μl^{-1}, 3 mM $MgCl_2$, 400 µM each dNTP), 1.0 µl of each primer (5 pmol of each one), and 9.5 µl of PCR-grade water (Sigma-Aldrich, U.S.A.). For BG12 amplification, PCR was performed using an initial denaturation at 95°C for 2 min, followed by 35 cycles of 30-s denaturation at 94°C, 30-s annealing at 57°C, 60-s extension at 72°C, and a final extension of 5 min at 72°C. To visualize the amplicons, 5 µl of each PCR product were visualized on a 2.0% agarose gel in 0.5× TAE buffer at 60 V for 1 h. The amplified PCR products were purified and sequenced by the company MACROGEN Inc. (South Korea). The identification of the VNTR sequences obtained were compared with other VNTR sequences deposited and available in the public GenBank databases (https://www.ncbi.nlm.nih.gov/) and using the nucleotide basic local alignment search tool program (BLASTn) (https://blast.ncbi.nlm.nih.gov). The number of different alleles considering the variations in the microsatellite tandem repeats as well as the punctual mutations detected in the sequences were visualized using the MEGA X program (Kumar et al. 2018).

Phenotypic characterization of *B. glumae* strains

Nineteen *B. glumae* strains were selected according to VNTR haplotypes and their geographic origin. To produce inoculum, the strains were grown in Tryptic Soy Broth (TSB) (Granucult, Millipore, Sigma Aldrich) at 150 rpm and incubated overnight at 28°C, and the cells were harvested by centrifugation at 13,000 rpm for 3 min (Bach et al. 2017) and washed with at least a 0.85%-sterile physiological saline solution twice. The cell suspension was standardized to an $OD_{600\ nm}$ of 1.0.

Pigment production. The toxoflavin production for each isolate was evaluated (Choi et al. 2021) by the intensity of the yellow coloration in a test tube containing King B media slant where it was initially streaked. Casamino acid-peptone-glucose (CPG) medium was used to visualize production of other pigments different to toxoflavine (Karki et al. 2012) containing 1.0 g of casamino acids, 10.0 g of peptone, 10.0 g of glucose, and 18.0 g of

agar in 1 liter of distilled water. The plates were incubated at 30°C for 7 days.

Production of cellulases and lipases. Cellulase production in *B. glumae* strains was evaluated in CMC Agar (Huang et al. 2012) containing 1.9 g of K_2HPO_4, 0.94 g of KH_2PO_4, 1.6 g of KCl, 1.43 g of NaCl, 0.15 g of NH_4SO_4, 0.037 g of $MgSO_4 \cdot 7H_2O$, 0.017 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of yeast extract, 10.0 g of carboxymethylcellulose, and 15.0 g of agar in 1 liter of distilled water at a pH of 7.2; the production of lipases was evaluated in agar Tween 80 (Slifkin 2000). The latter consisted of the mixture of solution A that contained 10.0 g of peptone, 5.0 g of NaCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 15.0 g of agar in 0.9 liters of distilled water, while solution B contained 10.0 g of Tween 80 in 0.1 liter of distilled water at a pH of 6.8. Solutions A and B were sterilized separately, combined at 50°C, and served in Petri dishes. In both trials, the strains were inoculated at a rate of 10 µl incubated at 28°C for 7 days and tested in triplicate. To demonstrate the cellulase activity, the Petri dishes were flooded with a solution of Congo red (1 mg/ml) for 15 min and then with a 1 M NaCl solution for 15 min. The presence of a yellow zone of hydrolysis around the colony was positive for the production of cellulases, reporting the absence or presence of a hydrolytic halo. For lipase activity, the presence of an opalescent halo around the colony, viewed in transmitted light, indicated a positive assay. The diameter of the colony and the opalescent halo were measured using a digital vernier (Dasqua, U.K.) to calculate the efficiency in the production of lipases using the following formula:

$$\%\ \text{Lipases} = ([\text{Opacity halo diameter} - \text{Colony diameter}]/\text{Colony diameter}) \times 100$$

Swarming and swimming capacity. The swarming and swimming motility of the *B. glumae* strains was evaluated according to Kim et al. (2018). *B. glumae* strains were grown overnight in LB Broth until reaching a final $OD_{600\ nm}$ = 0.5, and then they were seeded in each of the media under evaluation. The LB medium (Hi-media, India) supplemented with 0.3 and 0.7% agar, was inoculated at a rate of 5 and 1 µl to evaluate swarming and swimming, respectively. LB supplemented with 1.5% was used as control treatment. After the incubation period at 37°C for 24 h, the distance of bacterial growth compared with the control treatment was measured.

Virulence level assays

Maceration area on onion bulb scales. We estimated the virulence of strains by estimating the maceration area induced by each isolate on onion bulb. Karki et al. (2012) showed that there was a significant correlation between the abilities of *B. glumae* strains to cause maceration on onion bulb scales and to produce symptoms on rice panicles. The strains under study were cultivated in 3 ml of TSB broth and incubated at 28°C at 170 rpm for 24 h. The fermented broth

Fig. 2. *Burkholderia glumae* infection severity scale in rice seeds.

was centrifuged at 10,000 rpm for 3 min to harvest the cells. The cell pellet was washed with a sterile 10 mM $MgSO_4$ solution at least twice at 10,000 rpm for 5 min. The cell suspension was adjusted to an $OD_{600\ nm}$ of 0.1, and counting was performed using the drop plate method (Miles et al. 1938). White onion was used to obtain bulb scales. Each bulb scale was inoculated with 5 µl of cell suspension according to the experimental design of 21 treatments, 19 strains of *B. glumae*, one control treatment inoculated only with 10 mM $MgSO_4$ solution, and one negative control using the beneficial bacterium *Rhizobium leucaenae* CFN 299^T. The inoculated onion bulb scale was placed in Petri dishes with four replicates per treatment in each dish and incubated at 30°C for 48 h. The maceration area was calculated using a digital vernier (Dasqua, U.K.) and expressed in mm^2. The experiments were performed in triplicate, with 12 (three times four) measures per treatment.

Evaluation of the severity of the infection in two cultivars of rice. The adapted methodology of Flórez Zapata and Uribe Vélez (2011) was used to evaluate the severity of the virulence on two cultivars of rice. The strains were cultivated in 5 ml of TSB broth and incubated at 28°C at 170 rpm for 24 h. The fermented broth was centrifuged at 10,000 rpm for 3 min to obtain a cell pellet, which was washed with a sterile 10 mM $MgSO_4$ solution at least twice at 10,000 rpm for 5 min. The cell suspension was adjusted to an $OD_{600\ nm}$ of 0.5 (~10^8 CFU/ml), and the respective decimal dilutions were made to obtain a cell suspension of ~10^5 CFU/ml. Cell counts were performed using the drop plate method (Miles et al. 1938). Two rice cultivars were used, INIA 507 'La Esperanza' (LE) and INIA 510 'La Conquista' (LC), and are described as supposedly susceptible and moderately resistant to bacterial panicle blight by *B. glumae*, respectively. The seeds were selected, disinfected with 5% sodium hypochlorite solution for 8 min followed by 70% ethanol for 6 min, and rinsed at least six times in sterile distilled water. Finally, they were soaked for 24 h at room temperature. The seeds were immersed in each cell suspension at ~10^5 CFU/ml for 30 min under constant agitation (170 rpm) and left in sterile Petri dishes for 15 min. The experimental design consisted of 20 treatments (19 strains of *B. glumae* and one control treatment without inoculation), with four repetitions. Each replicate consisted of 20 seeds sown in magenta boxes (Chicago, U.S.A.) containing sterile moistened absorbent paper. The experiment was incubated at 25 ± 2°C for 7 days. The effect of inoculation on the germination of two rice cultivars was evaluated by recording the root length (mm), coleoptile length (mm), and total length (mm) of the seedlings. The severity of the infection in seeds was evaluated using the modified scale of Devescovi et al. 2007: level 1 = seedlings completely green and as vigorous as the uninoculated control, level 2 = seedlings completely green but with less vigorous roots and aerial parts compared with the uninoculated control, level 3 = seedlings with development of the aerial part of the plant, with partial decoration on less than 50% of the surface, level 4= seedlings with development of the aerial part of the plant, with partial decoration on more than 50% of the surface, level 5 = seedlings with development of the aerial part of the plant, with complete discoloration or with growth limited to less than 1 cm in height, and level 6 = macerated coleoptile and plumule without plant development (Fig. 2).

Statistical analyses

Significant differences between strains for their effects on seeds germination (coleoptile and radicle length) were tested by an analysis of variance (ANOVA) using the R command package in R Study Desktop version 1.3.1093. The normality and homoscedasticity of variances were tested. According to tests results, we either applied a Tukey test or a Kruskal-Wallis test, both with a significance level of 95%. We estimated the R-square correlation between measures (maceration areas, coleoptile and radicle length, and production of lipase) using a linear model function implemented in R. A Wilcoxon signed-rank test based on the maceration areas and the severity index of each strain was performed to search for significant correlation between our tests of virulence.

Results

Isolation and characterization of strains

We collected 132 grain samples of cultivated rice cultivars from four rice valleys: Bajo Mayo (30), Alto Mayo (57), Central Huallaga (15), and Alto Huallaga (30) (Table 1; Fig. 1).

The isolates obtained from grains were categorized as possible phytopathogenic *Burkholderia*, where 75 isolates presented typical growth characteristics of *B. glumae* or *B. gladioli*. However, toxoflavin production was variable among the isolates, with 5.33% presenting high toxoflavin production, 17.33% presenting medium production, 38.67% presenting low production, and 38.67% presenting no evidence of production (Table 2; Fig. 3). The standard strain of *B. glumae* B3HT presented high toxoflavin production.

Molecular discrimination of *Burkholderia*: *rec*A, *tox*B, and BOX-PCR genomic profiles

Only 47 isolates (61.33%) and the standard *B. glumae* B3HT strain from the collection of grain isolates resulted in a positive amplification for both the *rec*A and *tox*B genes. We used these positive amplification results to select the strains to be analyzed by the BOX-PCR profiling approach.

Table 2. Toxoflavin production in King B media (37°C by 48 h) of isolates from rice grains collected from four rice valleys in the San Martin region[z]

Strain code	Toxoflavine production	Strain code	Toxoflavine production
F1	+++	F37	–
F2	+	F38	–
F3	–	F39	–
F4	–	F40	++
F5	+	F41	+++
F6	–	F42	+
F7	+	F43	+
F8a	+	F44	+
F8b	–	F46a	+
F9	+	F46b	–
F10	+	F47	+
F11a	++	F48	–
F11b	+++	F49	++
F12a	–	F50a	–
F13a	++	F50b	+
F13b	+	F51	++
F14a	++	F52a	++
F15	++	F55	–
F16	+	F56	+
F17	–	F57	–
F18b	–	F58	++
F19	++	F60B	+
F20	–	F61	+
F21	+	F62	+
F22	++	F63	++
F23	–	F66	++
F24	–	F67a	+
F25a	–	F69b	+++
F25b	–	F70	+
F27	–	F71	+
F28	–	F72	+
F29	–	F73	+
F30	–	F74	+
F31	–	F75a	–
F32	–	F75b	+++
F34	+	F76	+
F35	+	F77	–
F36	+	F80	–
Burkholderia glumae B3HT			+++

[z] +++ = high toxoflavin production, ++ = medium production, + = low production, and – = no evidence of production.

The fragments obtained ranged from 500 to 10,000 bp. The fragments sizes were used to construct a similarity dendrogram. Considering a 90% similarity level as the cut-off point, 38 different groups were obtained. These groups were named G1 (BOX Group 1) to G38 (BOX Group 38), reflecting a high genotypic diversity among the phytopathogenic *Burkholderia* isolates (Fig. 4). The dendrogram consisted of 31 groups made up of a single strain; four groups of two strains made up of G5, G6, G28, and G32 of phytopathogenic *Burkholderia*; and three groups with three strains (G18, G23, and G36) including phytopathogenic *Burkholderia* strains. The standard strain used, *B. glumae* B3HT, belonged to the G18 group. The valley with the highest number of BOX-PCR groups (20) was Alto Huallaga, encompassing 52.63% of all BOX-PCR groups reported, followed by the Bajo Mayo valley with 14 BOX-PCR groups (36.84%) and four BOX-PCR groups (10.53%) belonging to the Huallaga Central valley. Although the same number of isolates from each valley (15) were used, we did not detect any clustering isolates originating from different geographic valleys. Although, strains F21 (Rosanaico Sector) and F14a-F16 (Estación El Porvenir Sector), belonging to G36, were sampled from different sites but still belong to the Bajo Mayo valley. Based on the BOX-PCR dendrogram, 38 representative isolates from the different BOX groups formed were selected for sequencing *rec*A and *tox*B genes. We detected three different *rec*A haplotypes along the 568- to 621-bp sequences obtained (variation of length due to quality of the sequences obtained). There is no correlation between the clusters obtained with the BOX-PCR analysis and the distribution in the dendogram of the different haplotypes, i.e., all isolates with the same haplotype do not cluster in the same clade. All *tox*B sequences (423 bp) were strictly identical. The gene sequences were deposited in GenBank with accession numbers for *rec*A (OQ706276 to OQ706313) and *tox*B (OQ706319 to OQ706356).

VNTR diversity in *B. glumae*

We detected seven different haplotypes among the 38 isolates amplified and sequenced for their VNTR locus, considering the number of repeats and single nucleotide polymorphisms (SNPs) in the two sequences that frame the repeats. The number of repeats of the 6-bp motif sequence ranged from two to 11. The number of isolates for each haplotype ranged from one (F49, haplotype 65, and F66, haplotype 7) to 17 (haplotype 4). In the same way as BOX-PCR clustering, there is no correlation between the geographical origin of the isolates and their VNTR haplotype, reflecting the within-valley diversity and potential dispersal of the bacterium among valley and among sites within each valley. While VNTR locus are usually considered as highly polymorphic, several haplotypes detected in our study have been previously detected in other studies and other countries. For instance, the 12 repeat types in our study display an identical sequence (including the 390-bp sequence framing the VNTR) with an isolate from Colombia (Béna et al. 2021). For the four repeat types, the same number of repeats was previously detected in other isolates but with several different SNPs on the sequence that frame the VNTR locus. The VNTR sequences were deposited in GenBank with accession numbers (OQ914559 to OQ914596).

Phenotypic characterization of *B. glumae* strains

The *B. glumae* strains produced various pigments in the CPG medium, highlighting the production of fluorescent green diffusible pigments in 36.84% of the strains. Other less-frequent pigments were gray, dark brown, and orange to dark orange (Table 3; Fig. 5); 99.47% of the *B. glumae* strains presented cellulase activity, and all the strains presented lipase activity, with a high level of variation among isolates, ranging from 30.16 (F410) to 133.33 (F7). Regarding swarming-type motility, 78.95% presented this type of

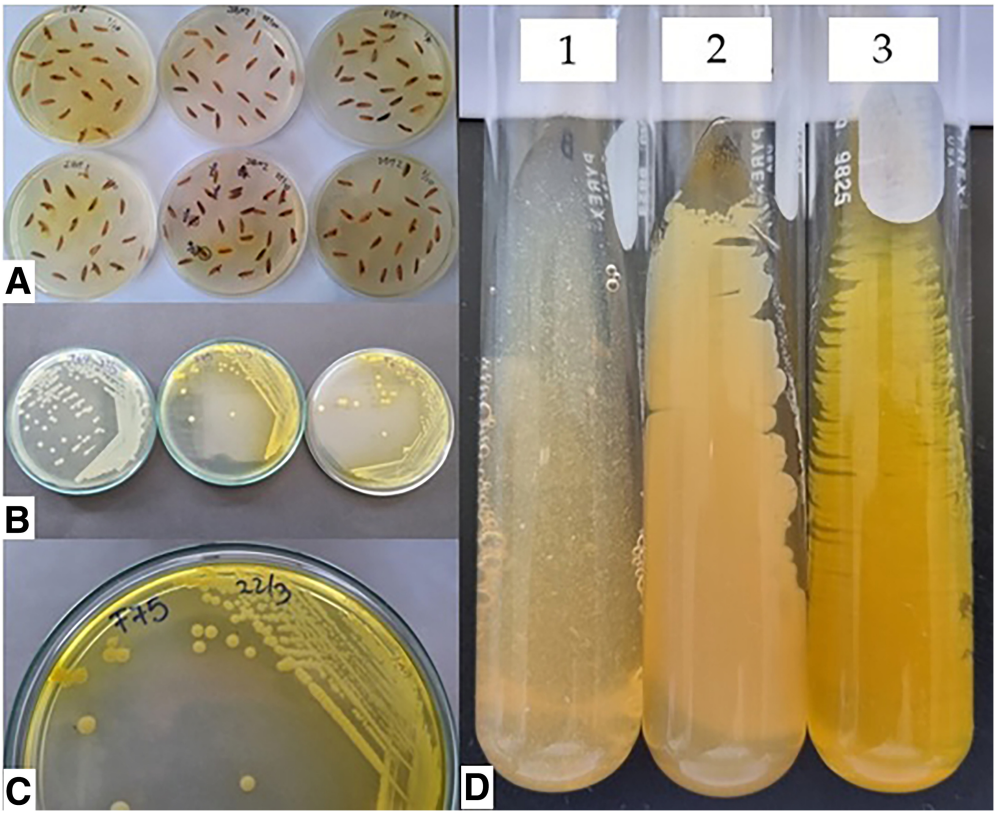

Fig. 3. A, Isolation of *Burkholderia glumae* from grains in modified CCNT medium and incubated at 41°C for 96 h; note the presence of the yellow fluorescent pigment toxoflavin around some grains. **B,** Purification of strains in Tryptic Soy Broth (TSB) medium incubated at 30°C for 48 h. **C,** Pure culture of *B. glumae* F75. **D,** Evaluation of toxoflavin production in Agar King B medium incubated at 30°C for 48 h, (1) without inoculation, (2) with *B. glumae* F67a (–), and (3) with *B. glumae* F31 (++).

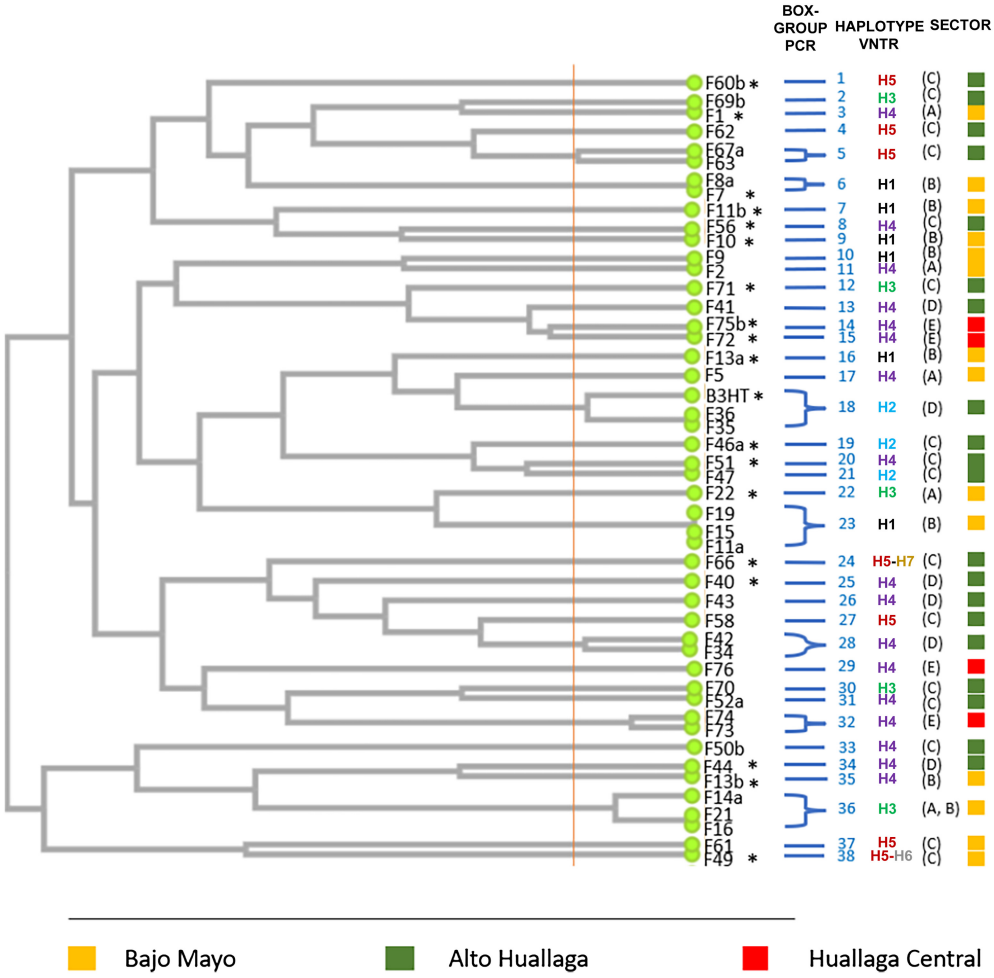

Fig. 4. Fingerprinting dendrogram of similarity based on the BOX-PCR profiles of the isolates of this study, using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and the Jaccard coefficient with 2% tolerance using the online dendrogram construction utility DendroUPGMA (http://genomes.urv.es/UPGMA/; Garcia-Vallvé et al. 1999). The letters in parentheses represent the sectors where the strains were isolated, and the valleys are represented by squares of different colors, according to Table 1. *Strains selected for virulence studies, according to their geographical origin.

Table 3. Phenotypic characterization of *Burkholderia glumae* strains isolated from the San Martin region

Strain (BOX-PCR group)	Pigment production on CPG[w]	Production of cellulases[x]	Efficiency in the production of lipases[y]	Motility swarming[z]	Motility swimming[z]
F60b (G1)	–	+	40.53 (±7.57) g	+	–
F1 (G3)	Fluorescent green (D)	+	40.15 (±3.58) g	–	–
F7 (G6)	Light grey (ND)	+	133.33 (±4.81) a	++	–
F11b (G7)	Fluorescent green (D)	+	63.89 (±2.78) defg	+	–
F56 (G8)	Grey (ND)	+	97.69 (±11.98) abcde	++	–
F10 (G9)	Fluorescent green (D)	+	40.60 (±1.07) g	+	–
F71(G12)	Dark brown (ND)	+	49.18 (±7.50) fg	++	–
F75b (G14)	Fluorescent green (D)	–	113.89 (±13.89) ab	–	–
F72 (G15)	–	+	103.96 (±10.02) abcd	+++	–
F13a (G16)	–	–	62.73 (±4.48) efg	+	–
B3HT (G18)	–	+	84.52 (±3.83) bcdef	–	–
F46a (G19)	Light grey (ND)	+	132.87 (±13.64) a	++	–
F51 (G20)	Orange (ND)	+	69.45 (±2.78) cdefg	+	–
F22 (G22)	Dark orange (ND)	+	109.56 (±13.59) abc	+	–
F66 (G24)	Fluorescent green (D)	+	61.11 (±2.78) efg	+	–
F40 (G25)	Fluorescent green (D)	+	30.16 (±1.59) g	+	–
F44 (G34)	Light grey (ND)	+	49.03 (±2.81) fg	++	–
F13b (G35)	Fluorescent green (D)	+	62.39 (±6.44) efg	+	–
F49 (G38)	–	+	52.74 (±4.51) fg	–	–

[w] CPG = Casaminoacids Peptone Glucose agar; – = absence of pigment production; D = diffusible pigment; and ND = nondiffusible or colony-associated pigment.
[x] Production of cellulases: + = formation of hydrolysis halo, and – = absence of hydrolysis halo.
[y] CV (%): 17.82. Values followed by different letters indicate significant differences (Tukey test, $P = 0.05$, and $n = 4$).
[z] Swarming and swimming motility: – = no motility, and +, ++, and +++ = increasing levels of swarming or swimming motility.

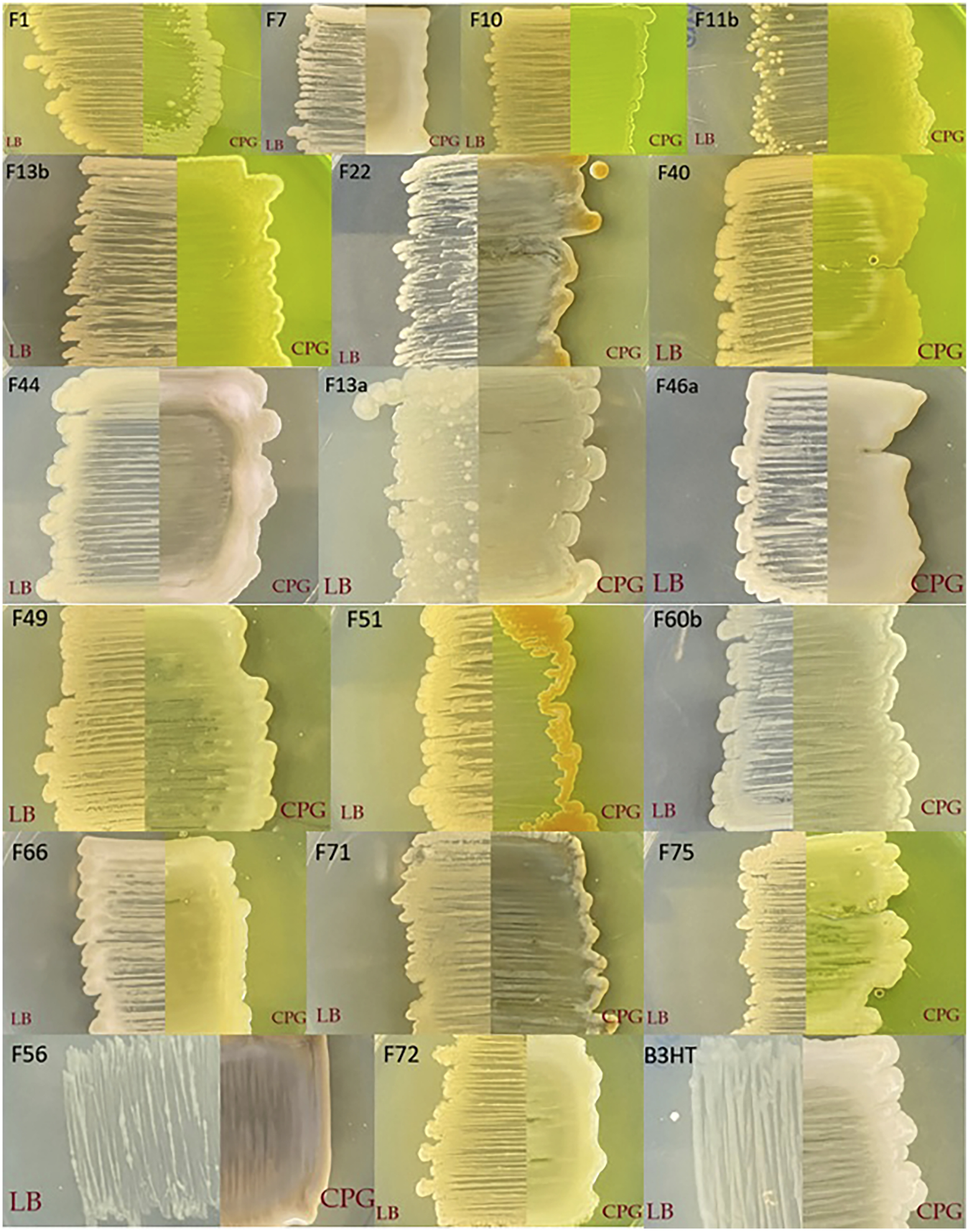

Fig. 5. Diversity of pigments produced by *Burkholderia glumae* strains on Luria-Bertani (LB) medium and casamino acid-peptone-glucose (CPG) medium. The photographs were obtained after 7 days at 28°C.

motility, with *B. glumae* F72 standing out. None of the strains presented swimming-type motility (Table 3).

Evaluation of the severity of the infection in two cultivars of rice

All the strains of *B. glumae* showed maceration activity in onion bulb scale, with strain F49 reaching a maximum maceration area of 189.40 mm^2 (Table 4). The strain *R. leucaenae* CFN 299^T, used as a control strain, did not present any maceration of onion bulb scale, therefore confirming it as a bacterium without any phytopathogenic capacity. In our tests, variations in maceration levels do not appear to be correlated with the estimated level of toxoflavin production. Thus, strains with a low level of production show large maceration surfaces (as for strains F10 and F56, see Tables 2 and 4). Conversely, we observed a positive correlation between the levels of diffusible pigmentation (D) observed for each strain in CPG medium and its ability to induce maceration of the onion bulb ($r = 0.32$) (Tables 3 and 4). The strains with the smallest maceration surface area show none or very little in CPG medium. In contrast, all strains (except strain F49) with the largest maceration surface area also showed strong pigmentation in the CPG medium (Fig. 5).

The virulence of *B. glumae* strains was also evaluated by measuring root length (mm), coleoptile length (mm), and total seedling length (mm) in two rice cultivars (Table 5; Fig. 6). The control treatment presented a normal development compared with the treatments inoculated with *B. glumae* strains. Regarding the total length, the percentage of growth inhibition in the LE cultivar ranged from 15.42 (F44) to 55% (F40), and the LC cultivar ranged from 26.47 (F72) to 51.14% (F51), both in comparison with the non-inoculated control treatment.

The correlation between maceration areas and severity index is weak ($R^2 = 0.13$; F-stat = 3.776, P value = 0.06874), while correlation between maceration areas and production of lipase was close to zero. Similarly, the Wilcoxon signed-rank test between rank for maceration areas and rank for severity indexes was not significant. While we logically observe a correlation between radicular and coleoptile lengths in each of the two rice cultivars ($R^2 = 0.45$ and 0.417 for LC and LE, respectively), there is no correlation between these lengths and lipase production of each strain.

Regarding the severity level of the *B. glumae* strains, there were slight differences between strains (Fig. 6). For instance, the percentage of seeds with level 2 infection severity ranged from 5.26 to 53.25% and 2.56 to 71.79% in LC and LE cultivars, respectively. The same ranges of variability are observed for severity indexes level 3 and 4. Seeds with a severity index of level 5 (complete discoloration or growth limited to less than 1 cm in height) and 6 (maceration of coleoptile and plumule) are much rarer, except for the strain F56 presenting the higher number (more than 10% of the seeds affected). To remain within acceptable test conditions, we performed a Chi-2 test with the data from the three severity scales greater than 5, i.e., levels 2 to 4 (Fig. 2). The test shows that symptom severity varies significantly between strains, revealing significant differences in pathogenicity between strains (X-squared = 258.58, df = 36, P value < $2.2e^{-16}$).

Table 4. Virulence characterization of *Burkholderia glumae* strains by maceration area estimate on onion bulb[z]

Strain (BOX-PCR group)	Maceration area (mm^2)
F60b (G1)	19.40 (±1.20) cde
F1 (G3)	117.30 (±13.08) bc
F7 (G6)	83.75 (±9.58) bcde
F11b (G7)	99.70 (±13.26) bcd
F56 (G8)	120.70 (±20.19) ab
F10 (G9)	117.85 (±9.24) b
F71(G12)	104.10 (±8.50) bcd
F75b (G14)	114.80 (±11.74) bc
F72 (G15)	95.40 (±6.93) bcd
F13a (G16)	37.60 (±3.10) de
B3HT (G18)	47.00 (±3.60) cde
F46a (G19)	70.45 (±11.96) bcde
F51 (G20)	117.60 (±22.44) b
F22 (G22)	96.75 (±17.78) bcd
F66 (G24)	114.75 (±16.34) bc
F40 (G25)	117.40 (±17.16) bc
F44 (G34)	82.25 (±4.78) bcde
F13b (G35)	105.50 (±13.57) bcd
F49 (G38)	189.40 (±4.43) a
Rhizobium leucaenae CFN299^T	0

[z] Values followed by different letters indicate significant differences (Tukey test, $P = 0.05$, and $n = 4$).

Discussion

Rice interacts with a wide variety of bacteria, with these associations being beneficial and mutualistic (Bertani et al. 2016; Ji et al. 2014; Sitlaothaworn et al. 2023; Sun et al. 2008) but also pathogenic, such as those caused by *Pantoea* (Azizi et al. 2020), *Xanthomonas oryzae* (Quibod et al. 2020), or species of the genus *Burkholderia* (Ortega and Rojas 2021; Riera-Ruiz et al. 2018). Bacterial panicle blight, caused by *B. glumae*, has been reported for a long time in Asia (Goto and Ohata 1956) but also in several Latin American countries, including Panama (Nandakumar et al. 2007), Ecuador (Riera-Ruiz et al. 2014), Colombia (Zeigler and Alvarez 1989), and more recently Perú (Valdez-Nuñez et al. 2020). While this bacterial disease is very common throughout Latin America and can potentially cause massive yield losses of up to 75 % (Ortega and Rojas 2021), it is rather surprising that very few studies have focused on the genetic and virulence diversity of strains present in cultivated fields. While multiple virulence factors are involved in the bacterial pathogenesis of *B. glumae*, leading to a wide range of virulence levels (Karki et al. 2012), understanding the genetic diversity and structure of the pathogen population is crucial in devising disease management strategies.

B. glumae shows high genetic diversity in Perú

Based on BOX-PCR profiling and sequencing of a VNTR locus, coupled with virulence and phenotypic tests, our study detected a wide diversity of *B. glumae* strains sampled from the four valleys and seven different rice cultivars in the growing region of San Martín. In a previous study, we evaluated the genetic diversity of 15 Peruvian strains of *B. glumae* isolated from grains and stems of rice cultivars from three departments of Perú. These strains grouped into nine BOX genomic profiles (Valdez-Nuñez et al. 2020). Previous studies had demonstrated the ability of BOX-PCR to reveal diversity among *B. glumae* isolates. Karki et al. (2012) studied the genomic profiles of 23 virulent and avirulent strains of *B. glumae*, reporting the grouping of two main phyletic clades and several subclades. These isolates, collected from Texas, Louisiana, and Arkansas, were not grouped according to geographic origin but were dispersed on the dendrogram. Kumar et al. (2023) observed a similar pattern when studying the genetic diversity of 10 *B. glumae* isolates sampled in India from three different regions, with five BOX-PCR and two ERIC-PCR groups. BOX-PCR profiling mostly revealed genomic rearrangements, as well as a large-scale inversion rearrangement of genetic elements, which is a very common situation in the genus *Burkholderia* (Bochkareva et al. 2018; Mannaa et al. 2019).

Besides the hypothesis that these variations in the genome might allow strains to adapt to new environments (Lee et al. 2016), this high level of diversity observed repeatedly in different studies together with the present study suggests that the bacterium has been present in South America for a very long time, leaving open the possibility of local genetic diversification or that the region has undergone repeated introductions of different evolutionary lineages of the bacterium, possibly through the trade in seeds of the bacterium-infected rice. The very high diversity of this marker based on genomic rearrangements makes it difficult to draw any conclusions. The *rec*A and VNTR locus sequence data could help to clarify the issue. A recent study, carried out in a single field in Colombia, also suggested a very high level of diversity using VNTR typing (Béna et al. 2021). In

Table 5. Virulence characterization of *Burkholderia glumae* strains isolated from the San Martin region[x]

	Radicular length (mm)				Coleoptile length (mm)				Total length (mm)			
Strain (BOX-PCR group)	**LE[y]**	**%I**	**LC[z]**	**%I**	**LE[y]**	**%I**	**LC[z]**	**%I**	**LE[y]**	**%I**	**LC[z]**	**%I**
F60b (G1)	50.49 (±2.54) abc	15.20	35.61 (±1.95) efg	29.75	37.92 (±2.28) b	28.48	17.14 (±0.89) efg	59.83	88.42 (±3.58) bc	21.46	52.75 (±2.18) ef	43.49
F1 (G3)	47.79 (±2.67) abcdef	19.74	41.43 (±1.69) bcde	18.27	34.13 (±2.03) bcde	35.63	21.51 (±0.83) bcde	49.59	81.92 (±3.06) bcde	27.23	62.93 (±2.22) bcde	32.58
F7 (G6)	35.15 (±2.39) efghi	40.96	36.39 (±2.47) cdefg	28.20	21.57 (±0.78) klm	59.32	17.88 (±0.74) efg	58.08	56.72 (±2.80) gh	49.61	54.28 (±2.80) def	41.86
F11b (G7)	51.98 (±2.69) ab	12.71	40.16 (±2.06) bcdef	20.77	29.86 (±1.17) defgh	43.68	18.54 (±1.35) defg	56.54	81.84 (±3.69) bcde	27.30	58.70 (±3.14) bcde	37.12
F56 (G8)	32.90 (±2.84) ghi	44.75	34.58 (±2.40) fg	31.78	19.46 (±2.26) m	63.30	19.17 (±0.96) cdef	55.06	52.36 (±3.28) h	53.49	53.75 (±2.90) def	42.42
F10 (G9)	36.79 (±3.02) defghi	38.21	39.85 (±1.45) bcdef	21.39	30.77 (±1.88) cdef	41.96	15.89 (±0.64) fg	62.75	67.57 (±4.30) defgh	39.98	55.74 (±1.76) cdef	40.29
F71 (G12)	41.09 (±2.23) bcdefgh	31.00	40.13 (±1.79) bcdef	20.83	25.19 (±1.30) ijk	52.49	18.87 (±0.76) cdef	55.78	66.28 (±2.47) efgh	41.12	58.99 (±2.12) bcde	36.80
F75b (G14)	34.94 (±2.36) efghi	41.31	42.94 (±2.72) bcd	15.29	28.42 (±1.98) fghij	46.41	21.70 (±0.92) bcde	49.13	63.36 (±3.45) fgh	43.71	64.64 (±2.97) bcde	30.76
F72 (G15)	34.54 (±3.01) fghi	41.99	45.02 (±2.01) b	11.18	30.22 (±2.33) cdefg	43.01	23.62 (±0.91) bc	44.63	64.76 (±4.43) fgh	42.47	68.64 (±2.35) b	26.47
F13a (G16)	47.18 (±2.46) abcdefg	20.76	40.86 (±2.48) bcde	19.38	26.36 (±2.46) hij	50.29	20.20 (±1.20) bcdef	52.65	73.54 (±4.13) cdefg	34.67	61.06 (±2.71) bcde	34.59
B3HT (G18)	49.95 (±2.63) abcd	16.12	44.23 (±2.08) b	12.74	23.66 (±1.23) jklm	55.37	21.40 (±0.93) bcde	49.85	73.61 (±3.34) cdefg	34.61	65.62 (±2.40) bcd	29.70
F46a (G19)	38.25 (±2.93) cdefghi	35.76	38.21 (±1.30) cdefg	24.61	28.59 (±0.85) efghi	46.08	15.48 (±0.79) fg	63.71	66.84 (±3.28) efgh	40.62	53.69 (±1.56) def	42.48
F51 (G20)	33.59 (±2.67) ghi	43.60	31.87 (±2.42) g	37.12	20.06 (±1.65) lm	62.16	13.74 (±1.09) g	67.80	53.65 (±3.59) h	52.34	45.61 (±3.20) f	51.14
F22 (G22)	40.58 (±3.76) bcdefgh	31.85	40.34 (±2.14) bcdef	20.42	34.06 (±3.30) cdef	35.77	18.44 (±0.91) defg	56.78	74.64 (±3.09) cdef	33.69	58.78 (±2.50) bcde	37.03
F66 (G24)	59.05 (±3.33) a	0.83	41.19 (±1.73) bcd	18.75	34.59 (±1.94) bcd	34.76	20.04 (±0.73) bcdef	53.03	93.64 (±4.23) b	16.81	61.22 (±1.98) bcde	34.41
F40 (G25)	26.00 (±2.09) i	56.34	38.16 (±1.86) defg	24.71	24.66 (±1.53) ijkl	53.49	19.27 (±0.83) cdef	54.84	50.66 (±2.32) h	55.00	57.43 (±2.20) bcdef	38.48
F44 (G34)	57.39 (±2.84) a	3.62	37.03 (±2.98) defg	26.95	37.83 (±1.47) b	28.66	22.77 (±0.85) bcd	46.63	95.22 (±2.79) b	15.42	59.80 (±3.15) bcde	35.94
F13b (G35)	39.00 (±2.41) bcdefghi	34.51	43.65 (±1.84) bc	13.89	26.97 (±1.29) ghij	49.13	24.51 (±1.38) b	42.55	65.97 (±2.40) efgh	41.40	68.15 (±2.38) bc	26.99
F49 (G38)	48.17 (±2.11) abcde	19.10	34.79 (±2.84) defg	31.36	35.80 (±2.18) bc	32.48	19.43 (±0.95) cdef	54.46	83.98 (±2.24) bcd	25.40	54.22 (±3.34) def	41.92
Control treatment (-I)	59.54 (±1.83) a		50.69 (±1.15) a		53.03 (±1.97) a		42.66 (±0.97) a		112.57 (±2.76) a		93.35 (±1.75) a	

[x] Values followed by different letters indicate significant differences. LE = INIA 507 'La Esperanza' rice cultivar; LC = INIA 510 'La Conquista' cultivar. %I, percent infected.
[y] Tukey test, $P = 0.05$.
[z] Kruskal-Wallis test, $P = 0.05$.

our study we detected seven different VNTR haplotypes at a single locus. Among them, using a simple BLAST analysis in GenBank, we found 100% identical sequences for strains isolated from China, South Korea, Bangladesh, and the United States. The same similarity with isolates from other continents was observed with the three *rec*A haplotypes (that only diverged from one another by a single mutation). Although this should of course be confirmed by more sequences, these data already suggest that the diversity recovered in these restricted regions of Perú includes a diversity of isolates that have their closer relatives in another continent, reflecting long distance dispersals. Apart from the genetic diversity observed, the BOX-PCR typing results do not show any correlation between the geographical origin of the strains and their genetic diversity. This also suggests that, thanks to the highly polymorphic genetic marker BOX-PCR, there is frequent dispersal of bacteria between fields located in different geographical areas of Perú, homogenizing strains between sites. We do not yet know whether this homogenization occurs through seed exchange, irrigation water, or other mechanisms, but it reflects the high capacity of *B. glumae* to spread efficiently and the difficulty of preventing its spread within a limited geographical area.

This result reinforces the need for in depth studies on the genetic diversity of *B. glumae* both in America and in Asia, with the latter region generally being considered as the origin of the bacteriosis, to understand its epidemiology at a large scale.

There is a wide variation of pigment production and virulence activity between isolates

This genetic diversity of strains also reflects a diversity of levels of pathogenicity and virulence. We have investigated the diversity of virulence in our strains using two approaches, but the results have not always been consistent. Indeed, even if all *tox*B partial sequences obtained from these isolates were strictly identical, we found variations in toxoflavin production between strains, suggesting that these strains may have varying levels of virulence, since toxoflavin is a critical virulence factor in *B. glumae*. However, although there were clear differences between strains and the maceration area in onion bulb scale that they induced after inoculation, there was clear correlation with the level of production of toxoflavin. This result was quite disappointing since Karki et al. (2012) suggested a good correlation ($R^2 = 0.67$) between the abilities of *B. glumae* strains to cause maceration on onion bulb scales and to produce symptoms on rice panicles. Moreover, they showed that all virulent strains produced toxoflavin, as expected, while several virulent and avirulent strains also produced other unknown pigments on alternative growth media. The production of toxoflavin, although necessary, may not be sufficient to directly estimate the virulence of a strain at the maceration level of onion bulb scale. The variation in toxoflavin production could be an adaptive response to fluctuating environmental conditions, leading to variable conditions for disease emergence. The regulation of virulence determinants such as toxoflavin production

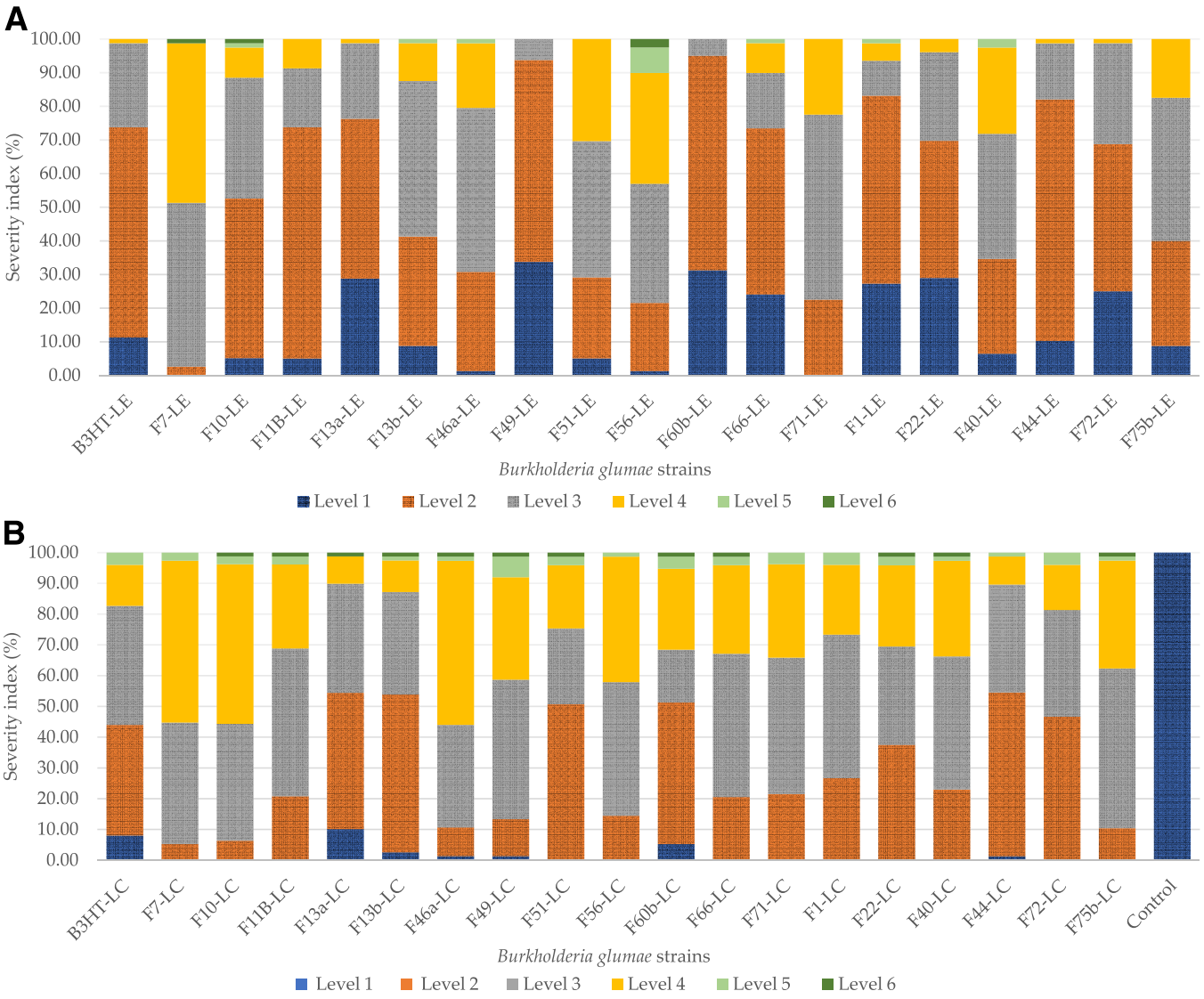

Fig. 6. Severity index of 19 strains of *Burkholderia glumae* in seeds of rice cultivar **A,** 'La Esperanza' (LE) and **B,** 'La Conquista' (LC).

and flagella is controlled by a quorum-sensing system (Ortega and Rojas 2021). This regulation may also be highly dependent on the mixing of different strains within natural populations, with complementation between isolates with different levels of virulence expression. Conversely, we found a surprising correlation between total pigment production on CPG medium and maceration level, specifically in diffusible pigments. This suggests that the pigments produced by the strains that are different from toxoflavin may play a role in the virulence of the strain or at least in onion maceration. Karki et al. (2012), who had also detected additional pigments, suggested the opposite, i.e., that other pigments do not play a role in the virulence of the strain. More detailed studies of the different pigments produced by these strains should be undertaken to explore the possibility that other compounds are involved in the virulence of the strains or not.

Rice cultivars show differences in terms of resistance to bacteria, particularly at the germination stage

In contrast to Karki et al. (2012), we found no correlation between the maceration area on the bulb and the effect of inoculation on radicular or coleoptile length, regardless of the rice variety tested. Karki et al. (2012) used previous greenhouse and field tests at the panicle stage to estimate the virulence of each strain on rice when we used seed inoculation and the first stages of development. However, as described by Pedraza et al. (2018) who used the same seed inoculation protocol, *B. glumae* maintains an endophyte population in seed-infected rice plants throughout the vegetative and reproductive stages. They also demonstrated that *B. glumae* was able to induce disease symptoms and reduce plant vigor when inoculated in seeds. Considering their results and ours, we must consider that although seed inoculation produces symptoms in radicle and coleoptile development and ultimately in the reproductive stage of the plant, virulence and pathogenesis mechanisms may not be identical or regulated in the same way at different stages of plant development. This would suggest that the selection of resistant rice cultivars or control methods against *B. glumae* should be carried out using different protocols depending on the different stages of plant development. The use of the two cultivars LE and LC was predetermined due to the supposed moderate resistance to bacterial panicle blight by the LC cultivar and the marked susceptibility of the LE cultivar. It is important to mention that this observation was made in evaluations at the field level (Edson E. Torres-Chávez, personal communication). Our data suggest the opposite, that the LC cultivar had higher severity levels for at least 84.2% of the strains under study, at least in the first stage of infection or establishment. Although more precise evaluations of resistance to the bacterium in the field should be carried out for both cultivars, this result is in line with previous findings, i.e., that the resistance mechanisms of rice at the early stages of seed germination could be different or even induce opposite effects to the mechanisms expressed at the later stages of development, again particularly during panicle establishment and grain filling. The need to carry out interactions between the highly diverse and virulent strains with the Peruvian rice cultivars under study is raised, with the aim of identifying useful combinations for future breeding programs in search of resistance to bacterial panicle blight.

The question of the genetic diversity of strains in adapting to environmental conditions is all the more crucial as the conditions under which the disease emerges and develops are still rather unclear, although the role of night temperature during the booting stage has been proposed (Echeverri-Rico et al. 2021; Ortega and Rojas 2021), showing that, at least in Colombia, the minimum temperature above 23°C during 10 days after flowering is the condition that correlates with disease incidence. Changing climatic conditions, with potential nighttime warming, coupled with the presence of a wide diversity of *B. glumae* strains, highlight the risk of a massive outbreak of the disease in Perú, with potentially dramatic consequences for food self-sufficiency. Although some studies have attempted to predict the onset of the disease using artificial intelligence (AI) models (Perez-Suarez et al. 2022), it appears that parameters such as the genetic diversity of the strains and the associated variable virulence and aggressiveness should be integrated.

Conclusions

High genetic diversity was observed among *B. glumae* isolates from a very small geographical area, as well as variability in virulence factors and severity of seed infection. Although no relationship was observed between the BOX-PCR genetic groups found and the geographical origin of the strains studied, the diversity found will allow us to understand and explain future changes in the epidemiology of bacterial leaf blight in the San Martin region. This information will be useful to complement the genomic approach carried out on the 19 *B. glumae* strains studied and will explain the phenotypic and virulence characteristics reported in the present study.

Acknowledgments

We thank the rice specialist Edson Torres Chávez for donating seeds of the La Esperanza and La Conquista varieties for the virulence and inoculation tests.

Literature Cited

Afzal, I., Shinwari, Z. K., Sikandar, S., and Shahzad, S. 2019. Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. Microbiol. Res. 221:36-49.

Arone, G., Calderón, C., Moreno, S., and Bedmar, E. J. 2014. Identification of *Ensifer* strains isolated from root nodules of *Medicago hispida* grown in association with *Zea mays* in the Quechua region of the Peruvian Andes. Biol. Fertil. Soils 50:185-190.

Azizi, M. M. F., Ismail, S. I., Ina-Salwany, M. Y., Hata, E. M., and Zulperi, D. 2020. The emergence of *Pantoea* species as a future threat to global rice production. J. Plant Prot. Res. 60:327-335.

Bach, E., Sant'Anna, F. H., Magrich dos Passos, J. F., Balsanelli, E., de Baura, V. A., Pedrosa, F. D. O., de Souza, E. M., and Passaglia, L. M. P. 2017. Detection of misidentifications of species from the *Burkholderia cepacia* complex and description of a new member, the soil bacterium *Burkholderia catarinensis* sp. nov. Pathog. Dis. 75:ftx076.

Bangratz, M., Wonni, I., Kini, K., Sondo, M., Brugidou, C., Béna, G., Gnacko, F., Barro, M., Koebnik, R., Silué, D., and Tollenaere, C. 2020. Design of a new multiplex PCR assay for rice pathogenic bacteria detection and its application to infer disease incidence and detect co-infection in rice fields in Burkina Faso. PLoS One 15:e0232115.

Béna, G., Fory, P. A., Rico, J. E., and Mosquera, G. M. 2021. VNTR typing of the bacterial rice pathogen *Burkholderia glumae* reveals the coexistence of several diverging lineages in a single field in Colombia. Plant Dis. 105: 3466-3473.

Bertani, I., Abbruscato, P., Piffanelli, P., Subramoni, S., and Venturi, V. 2016. Rice bacterial endophytes: Isolation of a collection, identification of beneficial strains and microbiome analysis. Environ. Microbiol. Rep. 8:388-398.

Bochkareva, O. O., Moroz, E. V., Davydov, I. I., and Gelfand, M. S. 2018. Genome rearrangements and selection in multi-chromosome bacteria *Burkholderia* spp. BMC Genom. 19:965.

Brown, A., and Smith, H. 2014. Benson's Microbiological Applications, Laboratory Manual in General Microbiology, Short Version. McGraw-Hill Education, New York City, NY.

Choi, O., Kim, S., Kang, B., Lee, Y., Bae, J., and Kim, J. 2021. Genetic diversity and distribution of Korean isolates of *Burkholderia glumae*. Plant Dis. 105: 1398-1407.

Cui, Z., Wang, S., Kakar, K. U., Xie, G., Li, B., Chen, G., and Zhu, B. 2021. Genome sequence and adaptation analysis of the human and rice pathogenic strain *Burkholderia glumae* AU6208. Pathogens 10:87.

Devescovi, G., Bigirimana, J., Degrassi, G., Cabrio, L., LiPuma, J. J., Kim, J., Hwang, I., and Venturi, V. 2007. Involvement of a quorum-sensing-regulated lipase secreted by a clinical isolate of *Burkholderia glumae* in severe disease symptoms in rice. Appl. Environ. Microbiol. 73:4950-4958.

Dobritsa, A. P., and Samadpour, M. 2016. Transfer of eleven species of the genus *Burkholderia* to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov. to accommodate twelve species of the genera *Burkholderia* and *Paraburkholderia*. Int. J. Syst. Evol. Microbiol. 66:2836-2846.

Echeverri-Rico, J., Petro, E., Fory, P. A., Mosquera, G. M., Lang, J. M., Leach, J. E., Lobaton, J. D., Garcés, G., Perafán, R., Amezquita, N., Toro, S., Mora, B., Cuasquer, J. B., Ramirez-Villegas, J., Rebolledo, M. C., and Torres, E. A. 2021. Understanding the complexity of disease-climate interactions for rice bacterial panicle blight under tropical conditions. PLoS One 16:e0252061.

Estrada-de Los Santos, P., Palmer, M., Chávez-Ramírez, B., Beukes, C., Steenkamp, E. T., Briscoe, L., Khan, N., Maluk, M., Lafos, M., Humm, E., Arrabit, M., Crook, M., Gross, E., Simon, M. F., Dos Reis Junior, F. B., Whitman, W. B., Shapiro, N., Poole, P. S., Hirsch, A. M., Venter, S. N., and James, E. K. 2018. Whole genome analyses suggests that *Burkholderia s*ensu lato contains two additional novel genera (*Mycetohabitans* gen. nov., and

Trinickia gen. nov.): Implications for the evolution of diazotrophy and nodulation in the *Burkholderiaceae*. Genes 9:389.

Faucher, S. P., Matthews, S., Nickzad, A., Vounba, P., Shetty, D., Bédard, É., Prévost, M., Déziel, E., and Paranjape, K. 2022. Toxoflavin secreted by *Pseudomonas alcaliphila* inhibits the growth of *Legionella pneumophila* and *Vermamoeba vermiformis*. Water Res. 216:118328.

Flórez Zapata, N. M. V., and Uribe Vélez, D. 2011. Determination of the infection of *Burkholderia glumae* in commercial Colombian rice varieties. Rev. Fac. Nac. Agron. Medellín 64:6093-6104.

Fonseca-Guerra, I., Fonseca-Guerra, G. A., and Oropeza, M. 2022. Bioassay for the in vitro pathogenicity evaluation of *Burkholderia gladioli* on *Solanum tuberosum* plants. Potato Res. 65:549-569.

Garcia, E. C., and Cotter, P. A. 2016. *Burkholderia thailandensis*: Growth and laboratory maintenance. Curr. Protoc. Microbiol. 42:4C.1.1-4C.1.7.

Garcia-Vallvé, S., Palau, J., and Romeu, A. 1999. Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in *Escherichia coli* and *Bacillus subtilis*. Mol. Biol. Evol. 16:1125-1134.

Goto, K., and Ohata, K. 1956. New bacterial diseases of rice (bacterial brown stripe and bacterial grain rot). Ann. Phytopathol. Soc. Jpn. 21:46-47 (abstract in Japanese).

Ham, J. H., Melanson, R. A., and Rush, M. C. 2011. *Burkholderia glumae*: Next major pathogen of rice? Mol. Plant Pathol. 12:329-339.

Huang, S., Sheng, P., and Zhang, H. 2012. Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). Int. J. Mol. Sci. 13:2563-2577.

Jaccard, P. 1912. The distribution of the flora in the alpine zone. New Phytol. 11: 37-50.

Ji, S. H., Gururani, M. A., and Chun, S.-C. 2014. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol. Res. 169:83-98.

Jin, Y., Zhou, J., Zhou, J., Hu, M., Zhang, Q., Kong, N., Ren, H., Liang, L., and Yue, J. 2020. Genome-based classification of *Burkholderia cepacia* complex provides new insight into its taxonomic status. Biol. Direct 15:6.

Jungkhun, N., Gomes de Farias, A. R., Watcharachaiyakup, J., Kositcharoenkul, N., Ham, J. H., and Patarapuwadol, S. 2022. Phylogenetic characterization and genome sequence analysis of *Burkholderia glumae* strains isolated in Thailand as the causal agent of rice bacterial panicle blight. Pathogens 11: 676.

Karki, H. S., Shrestha, B. K., Han, J. W., Groth, D. E., Barphagha, I. K., Rush, M. C., Melanson, R. A., Kim, B. S., and Ham, J. H. 2012. Diversities in virulence, antifungal activity, pigmentation and DNA fingerprint among strains of *Burkholderia glumae*. PLoS One 7:e45376.

Kawaradani, M., Okada, K., and Kusakari, S. 2000. New selective medium for isolation of *Burkholderia glumae* from rice seeds. J. Gen. Plant Pathol. 66: 234-237.

Kim, J., Mannaa, M., Kim, N., Lee, C., Kim, J., Park, J., Lee, H.-H., and Seo, Y.-S. 2018. The roles of two *hfq* genes in the virulence and stress resistance of *Burkholderia glumae*. Plant Pathol. J. 34:412-425.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Koeuth, T., Versalovic, J., and Lupski, J. R. 1995. Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. Genome Res. 5:408-418.

Kumar, S., Mondal, K. K., Ghoshal, T., Kulshreshtha, A., Sreenayana, B., Amrutha Lakshmi, M., Mrutyunjaya, S., Rashmi, E. R., Kalaivanan, N. S., and Mani, C. 2023. Genetic and pathogenic diversity analysis of *Burkholderia glumae* strains from Indian hot spot regions causing bacterial panicle blight of rice (*Oryza sativa* L.). Trop. Plant Pathol. 48:139-153.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35:1547-1549.

Lee, H.-H., Lelis, T., Ontoy, J., Bruno, J., Ham, J. H., and Seo, Y.-S. 2021. Complete genome sequence data of four *Burkholderia glumae* strains isolated from rice fields in the United States. Mol. Plant-Microbe Interact. 34:1324-1327.

Lee, H.-H., Park, J., Kim, J., Park, I., and Seo, Y.-S. 2016. Understanding the direction of evolution in *Burkholderia glumae* through comparative genomics. Curr. Genet. 62:115-123.

Lin, Q.-h., Lv, Y.-y., Gao, Z.-h., and Qiu, L.-h. 2020. *Pararobbsia silviterrae* gen. nov., sp. nov., isolated from forest soil and reclassification of *Burkholderia alpina* as *Pararobbsia alpina* comb. nov. Int. J. Syst. Evol. Microbiol. 70: 1412-1420.

Lopes-Santos, L., Castro, D. B. A., Ferreira-Tonin, M., Corrêa, D. B. A., Weir, B. S., Park, D., Ottoboni, L. M. M., Neto, J. R., and Destéfano, S. A. L. 2017. Reassessment of the taxonomic position of *Burkholderia andropogonis* and description of *Robbsia andropogonis* gen. nov., comb. nov. Antonie van Leeuwenhoek 110:727-736.

Mahenthiralingam, E., Bischof, J., Byrne, S. K., Radomski, C., Davies, J. E., Av-Gay, Y., and Vandamme, P. 2000. DNA-Based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. J. Clin. Microbiol. 38:3165-3173.

Mannaa, M., Park, I., and Seo, Y.-S. 2019. Genomic features and insights into the taxonomy, virulence, and benevolence of plant-associated *Burkholderia* species. Int. J. Mol. Sci. 20:121.

Miles, A. A., Misra, S. S., and Irwin, J. O. 1938. The estimation of the bactericidal power of the blood. J. Hyg. 38:732-749.

Nandakumar, R., Rush, M. C., and Correa, F. 2007. Association of *Burkholderia glumae* and *B. gladioli* with panicle blight symptoms on rice in Panama. Plant Dis. 91:767.

Nandakumar, R., Shahjahan, A. K. M., Yuan, X. L., Dickstein, E. R., Groth, D. E., Clark, C. A., Cartwright, R. D., and Rush, M. C. 2009. *Burkholderia glumae* and *B. gladioli* cause bacterial panicle blight in rice in the southern United States. Plant Dis. 93:896-905.

Ortega, L., and Rojas, C. M. 2021. Bacterial panicle blight and *Burkholderia glumae*: From pathogen biology to disease control. Phytopathology 111: 772-778.

Pedraza, L. A., Bautista, J., and Uribe-Vélez, D. 2018. Seed-born *Burkholderia glumae* infects rice seedling and maintains bacterial population during vegetative and reproductive growth stage. Plant Pathol. J. 34:393-402.

Perez-Suarez, J., Saboya, N., and Sullon, A. A. 2022. Prediction model of the *Burkholderia glumae* pest in rice crops using machine learning and spatial interpolation. Pages 681-694 in: Mobile Computing and Sustainable Informatics: Proceedings of ICMCSI 2022. Springer Nature Singapore, Singapore.

Quibod, I. L., Atieza-Grande, G., Oreiro, E. G., Palmos, D., Nguyen, M. H., Coronejo, S. T., Aung, E. E., Nugroho, C., Roman-Reyna, V., Burgos, M. R., Capistrano, P., Dossa, S. G., Onaga, G., Saloma, C., Cruz, C. V., and Oliva, R. 2020. The Green Revolution shaped the population structure of the rice pathogen *Xanthomonas oryzae* pv. *oryzae*. ISME J. 14:492-505.

Ramachandran, K., Vijaya, S. I., Ahmad, F. N., Amzah, B., and Zakaria, L. 2021. Characterization and identification of *Burkholderia glumae* as the causal pathogen of bacterial panicle blight of rice (*Oryza sativa* L.) in Malaysian rice granaries. J. Gen. Plant Pathol. 87:164-169.

Riera-Ruiz, C., Castro-Lara, J., Jimenez-Feijoó, M. I., and Cevallos-Cevallos, J. M. 2018. Interactions of *Burkholderia glumae* and *B. gladioli* in symptom development in rice seeds and seedlings. Can. J. Plant Pathol. 40:347-357.

Riera-Ruiz, C., Vargas, J., Cevallos-Cevallos, J. M., Ratti, M., and Peralta, E. L. 2014. First report of bacterial panicle blight of rice caused by *Burkholderia gladioli* in Ecuador. Plant Dis. 98:1577.

Sawana, A., Adeolu, M., and Gupta, R. S. 2014. Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: Proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. Front. Genet. 5:429.

Sitlaothaworn, K., Budsabun, T., Booncharoen, A., Panphut, W., Savarajara, A., and Tanasupawat, S. 2023. Diversity of plant growth-promoting endophytic bacteria, genome analysis of strain Sx8-8 and its rice germination promoting activity. Microbiology 92:269-283.

Slifkin, M. 2000. Tween 80 opacity test responses of various *Candida* species. J. Clin. Microbiol. 38:4626-4628.

Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. W. H. Freeman, San Francisco, CA.

Somasegaran, P., and Hoben, H. J. 1994. Collecting nodules and isolating rhizobia. Pages 7-23 in: Handbook for Rhizobia: Methods in Legume-Rhizobium Technology. Springer, New York, NY.

Spilker, T., Baldwin, A., Bumford, A., Dowson, C. G., Mahenthiralingam, E., and LiPuma, J. J. 2009. Expanded multilocus sequence typing for *Burkholderia* species. J. Clin. Microbiol. 47:2607-2610.

Sun, L., Qiu, F., Zhang, X., Dai, X., Dong, X., and Song, W. 2008. Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. Microb. Ecol. 55:415-424.

Valdez-Nuñez, R. A., Ríos-Ruiz, W. F., Ormeño-Orrillo, E., Torres-Chávez, E. E., and Torres-Delgado, J. 2020. Genetic characterization of rice endophytic bacteria (*Oryza sativa* L.) with antimicrobial activity against *Burkholderia glumae*. Rev. Argent. Microbiol. 52:315-327.

Wang, M., Wei, P., Cao, M., Zhu, L., and Lu, Y. 2016. First report of rice seedling blight caused by *Burkholderia plantarii* in North and Southeast China. Plant Dis. 100:645.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., and Arakawa, M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36:1251-1275.

Zeigler, R. S., and Alvarez, E. 1989. Grain discoloration of rice caused by *Pseudomonas glumae* in Latin America. Plant Dis. 73:368.

Zhou, X.-G. 2019. Sustainable strategies for managing bacterial panicle blight in rice. In: Protecting Rice Grains in the Post-Genomic Era. Y. Jia, ed. IntechOpen, London, U.K.