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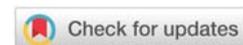
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## Research Article

# Bacterial blight of *Brachiaria* caused by *Burkholderia glumae* in Colombia

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## Abstract

A new disease of *Brachiaria* was observed in 2009 at the CIAT experiment station in Palmira, Colombia, on plants of *B. humidicola* (CIAT accession no.16888). In 2016, the disease was observed on multiple genotypes of *B. humidicola*, *Brachiaria* hybrid cv. Mulato II, and *Brachiaria* hybrid Cayman. Symptoms included chlorosis along the midribs and yellowing on flag-leaf margins, followed by wilting and necrosis of foliage. Bacteria isolated from the lesions were cream-colored and produced a yellow, diffusible, non-fluorescent pigment on King's medium B. Thirty-two bacterial strains fitting this description were pathogenic on *Brachiaria* spp. and were identified as *Burkholderia glumae* by PCR and sequence analysis of 16S rDNA. Real-time PCR was the most sensitive and accurate method evaluated for identifying the pathogen. *B. glumae* 88b, a highly-virulent strain identified in this study, was inoculated to ten *Brachiaria* genotypes including *B. decumbens*, *B. brizantha* 'Marandú', *B. brizantha* 'Toledo', *B. ruziziensis*, *B. brizantha* 'Piata' and *Brachiaria* hybrids CIAT 36061, CIAT 36062, CIAT 36087, BR02/1752, and BR02/1794. *B. glumae* 88b was pathogenic on nine of the *Brachiaria* genotypes; interestingly, CIAT 36062 was resistant to strain 88b. This knowledge of *B. glumae* would help to develop bacterial blight disease management.

## Introduction

Forage grasses in the genus *Brachiaria* are widely sown in cattle-producing regions in tropical Latin America [1,2] and are used by small-scale livestock producers in Africa and southeast Asia [3-5]. The most important commercial species include *B. brizantha*, *B. decumbens*, *B. ruziziensis*, and *B. humidicola*; these forage grasses are native to East Africa and were introduced to Latin America during the mid-20<sup>th</sup> century [6]. Since then, *Brachiaria* spp. have been planted on 99 million ha in Brazil alone [1], and their cultivation has drastically improved the efficiency of cattle production in areas with marginal or acid soils [2]. A recent study examined the economic impact of new *Brachiaria* cultivars in Brazil, Colombia and Central American countries and estimated their value at \$6.7 billion [7].

The bacterial genus *Burkholderia* consists of more than 40 different gram-negative species that occupy a wide array of ecological niches. *Burkholderia* spp. occur in soil, water, fungal mycelia, the plant rhizosphere, and as endophytes in roots and

shoots [8]. *B. glumae* (formerly *Pseudomonas glumae*), the causal agent of Bacterial Panicle Blight (BPB) in rice, causes economic losses in Korea, Japan, the southeastern United States and South and Central America, Africa, Vietnam, the Philippines, Taiwan, and other rice-growing areas worldwide [9-14]. Yield losses of 15% are common and losses up to 80% have been recorded in severely-infected rice fields [15]. *B. glumae* also cause seedling rot in nurseries [16] and has been identified as a causal agent of bacterial wilt in tomato, pepper, eggplant, sunflower and sesame [17]. *B. glumae* motility appears to contribute to pathogenesis as motility-deficient mutants have low virulence when inoculated in rice at the flowering stage. *B. glumae* is more prevalent and virulent than either *B. gladioli* or *B. plantarii* [18,19].

In Colombia, *B. glumae* was first reported to cause BPB in rice in 1987 [20]. Since then, the disease has been documented in many other rice-producing countries in Central and South America including Costa Rica, Cuba, the Dominican Republic, Ecuador, Nicaragua, Panama and Venezuela [21-24]. *B. glumae*

has not been previously reported in association with *Brachiaria* or other tropical forage grasses; however, the temperate forage grass *Lolium multiflorum* (Italian ryegrass) is a known alternate host for the pathogen [25]. *B. humidicola* is a known host for other *Burkholderia* spp. including *B. pseudomallei*, the causal pathogen of melioidosis in humans and animals in Northern Australia [26].

In 2009, a new disease of *Brachiaria* was observed on *B. humidicola* CIAT accession no. 16888 at the CIAT experiment station in Palmira, Colombia. The disease was observed again in 2016 on *B. humidicola* and *Brachiaria* hybrids. Symptoms included a chlorotic streak along the midribs and yellowing on flag-leaf margins, followed by wilting and necrosis of leaf tissue. Initial tests indicated that *B. glumae* was present in the foliage of the diseased plants. In the present study, we confirmed the pathogenicity of *B. glumae* in *Brachiaria* spp. Strains of *B. glumae* were identified using species-specific PCR primers and subsequent sequencing of 16S rDNA. This article constitutes a report of *B. glumae* as the causal agent of bacterial blight of *Brachiaria* spp.

## Materials and methods

This study was conducted in the laboratories and greenhouses of the Forage Pathology Program at CIAT, Palmira, Colombia (1001 masl; 3.5833 °N, 76.2500 °W).

### Isolation and purification of bacterial strains

Bacterial strains were isolated from diseased plants ( $n = 120$ ) of *B. humidicola*, *Brachiaria* hybrid cv. Mulato and *Brachiaria* hybrid Cayman. *Brachiaria* samples were collected in Palmira, Valle, in 2009 and 2016 (Table 1). The isolation of plant pathogenic bacteria was done by direct plating of infected leaf samples [27]. Small leaf fragments were washed under running tap water for 30 min, air dried on filter papers, and placed onto Nutrient Agar (NA) medium with the inclusion of a 5 min rinse in sodium hypochlorite (2% NaClO), used to sterilize the surface. Bacterial colonies were selected randomly from the disease samples for a detailed study. They included colonies with white growth typical of *B. glumae* and other commonly occurring colonies with cream-white growth.

**Table 1:** Identification of selected bacterial strains isolated from diseased *Brachiaria* plants from Colombia using different methods.

Strain no. <sup>a</sup>	Bacterial Species	Host Genotype No.	Host species	Origin of strain <sup>b</sup>	Colony pigment	Pathogenicity test <sup>c</sup>	PCR <sup>d</sup>	qPCR
<b><i>B. glumae</i> strains were isolated in the current study in 2009, 2010, and 2016.</b>								
98-36	<i>B. glumae</i>	CIAT 36087	<i>B.</i> hybrid 'Mulato II'	PV	+	++	+	18.10
99-37	<i>B. glumae</i>	CIAT 36087	<i>B.</i> hybrid 'Mulato II'	PV	+	++	+	18.40
98-43	<i>B. glumae</i>	CIAT 36087	<i>B.</i> hybrid 'Mulato II'	PV	+	++	+	20.30
97-44	<i>B. glumae</i>	CIAT 36061	<i>B.</i> hybrid 'Mulato'	PV	+	++	+	23.04
99-46	<i>B. glumae</i>	CIAT 36087	<i>B.</i> hybrid 'Mulato II'	PV	+	++	+	19.14
95-49	<i>B. glumae</i>	CIAT 36087	<i>B.</i> hybrid 'Mulato II'	PV	+	++	+	18.10
98-52	<i>B. glumae</i>	CIAT 36061	<i>B.</i> hybrid 'Mulato'	PV	+	++	+	17.5
95-55	<i>B. glumae</i>	CIAT 36061	<i>B.</i> hybrid 'Mulato'	PV	+	++	+	14.0
92E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	18.0
103E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	18.42
91E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	18.11
94E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	23.4
84E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	21.53
85E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	20.29
97-1E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	15.61
100E	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	14.13
88e	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	++	+	15.11
88b	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	PV	+	+++	+	16.29
10	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> . Hybrid Cayman	PV	+	++	+	15.46
4	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	19.14
6	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	17.5
15	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	18.00
21	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	19.53
19	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	16.48



45	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	17.24
61	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	15.61
70	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> hybrid Cayman	PV	+	++	+	16.29
96	<i>B. glumae</i>	BR02/1752	<i>Brachiaria</i> . Hybrid Cayman	PV	+	++	+	19.35
BG1	<i>B. glumae</i>	CIAT 16888	<i>Brachiaria humidicola</i>	Colombia	+	+	+	(+) ct=18.11
BG3	<i>B. glumae</i>	CIAT 16889	<i>Brachiaria humidicola</i>	Colombia	+	+	+	(+) ct=18.42
BG5	<i>B. glumae</i>	CIAT 16890	<i>Brachiaria humidicola</i>	Colombia	+	+	+	(+) ct=20.29
E93	<i>B. glumae</i>	CIAT 16891	<i>Brachiaria humidicola</i>	Colombia	+	+	+	(+) ct=23.04

**Reference strains were used in the current study.**

3845-5	<i>B. gladioli</i>	Rice	<i>Oryza sativa</i>	Colombia	+	NT	+	Nct
3161-2 (BG)	<i>B. glumae</i>	Rice	<i>Oryza sativa</i>	Colombia	+	NT	+	(+) 19.14
3193-4 (BG)	<i>B. glumae</i>	Rice	<i>Oryza sativa</i>	Colombia	+	NT	+	(+) 18
3193-5 (BG)	<i>B. glumae</i>	Rice	<i>Oryza sativa</i>	Colombia	+	NT	+	(+) 17.5
3252-8	<i>B. glumae</i>	Rice	<i>Oryza sativa</i>	NA	+	NT	+	16.48
3459-4 (BG)	<i>B. glumae</i>	Rice	<i>Oryza sativa</i>	Colombia	+	NT	+	(+) 15.46
3845-5	<i>B. gladioli</i>	Rice (Fedearroz 174)	<i>Oryza sativa</i>	VM	+	NT	+	Nct
<i>P. marginalis</i>	<i>P. marginalis</i>	Pathology CIAT	<i>Oryza sativa</i>	Colombia	-	-	-	Nct
<i>P. putida</i>	<i>P. putida</i>	Pathology CIAT	No data	Colombia	-	-	-	Nct
<i>P. stewartii</i>	<i>P. stewartii</i>	Pathology CIAT	<i>B. ruziziensis</i>	Colombia	-	-	-	Nct
<i>X.campestris</i> pv. <i>Graminis</i>	<i>X.campestris</i> pv. <i>Graminis</i>	Pathology CIAT	<i>Brachiaria</i>	Colombia	-	-	-	Nct
<i>B. glumae</i> KP689102.1	<i>B. glumae</i>	NCBI sequence	<i>Oryza sativa</i>	India	NT	NT	NT	NT
ATCC®33664™	<i>B. gladioli</i>	NCBI sequences with ATCC	No data	Japan	NT	NT	NT	NT
ATCC®12633™	<i>Pseudomonas putida</i>	NCBI sequences with ATCC	No data	USA	NT	NT	NT	NT
ATCC®19302™	<i>B. gladioli</i>	NCBI sequences with ATCC	No data	Japan	NT	NT	NT	NT
ATCC®25416™	<i>B. cepacia</i>	NCBI sequences with ATCC	Human	Japan	NT	NT	NT	NT
ATCC®25418™	<i>B. caryophylli</i>	NCBI sequences with ATCC	No data	Japan	NT	NT	NT	NT
ATCC®19311™	<i>B. andropogonis</i>	NCBI sequences with ATCC	No data	Japan	NT	NT	NT	NT
ATCC®51545™	<i>B. plantarii</i>	NCBI sequences with ATCC	No data	Japan	NT	NT	NT	NT
ATCC®10844™	<i>P. marginalis</i>	NCBI sequences with ATCC	No data	Japan	NT	NT	NT	NT
ATCC®10771™	<i>X. translucens</i>	NCBI sequences with ATCC	No data	USA	NT	NT	NT	NT
ATCC®33804™	<i>X. translucens</i>	NCBI sequences with ATCC	<i>Poa trivialis</i>	USA	NT	NT	NT	NT
ATCC®8199™	<i>P. stewartii</i>	NCBI sequences with ATCC	No data	Korea	NT	NT	NT	NT
ATCC®BAA-247™	<i>B. multivorans</i>	NCBI sequences with ATCC	Human	USA	NT	NT	NT	NT
ATCC®17400™	<i>P. fluorescens</i>	NCBI sequences with ATCC	<i>Arabidopsis thaliana</i>	France	NT	NT	NT	NT
ATCC 33617	<i>B. glumae</i>		<i>Oryza sativa</i>	Japan	NT	NT	NT	NT
ATCC 19302	<i>B. gladioli</i>		Onion	USA	NT	NT	NT	NT
ATCC 43733	<i>B. plantarii</i>		<i>Oryza sativa</i>	Japan	NT	NT	NT	NT
ATCC 10856	<i>B.cepacia</i>		-		NT	NT	NT	NT

<sup>a</sup>Bacterial strains were isolated from either leaves or seeds. ATCC = American Type Culture Collection (Manassas, VA, USA)

<sup>b</sup>Origin of strain: PV = Palmira, Valle; VM = Villavicencio, Meta, NA = Nechí, Antioquia; NT = not tested

<sup>c</sup>+, ++, +++, refer to weakly, moderately, and highly virulent reaction. - = non-pathogenic; NT = not tested for the respective analysis

<sup>d</sup>All the strains were screened with *B. glumae* primers, followed by other primers if not identified (*B. gladioli*). (-) = Negative, (NT) = no PCR performed; Nct = Negative

Isolates were originally isolated onto NA, transferred to King B and Miyajima's medium (KB), maintained on 50% glycerol and kept in storage at  $-20^{\circ}\text{C}$  before testing. The isolates were observed under Near Ultra Violet (NUV) light (under a UV lamp) and a loopful of non-fluorescing bacterial colonies were transferred to Miyajima's medium and incubated for 2 days at  $27^{\circ}\text{C}$ . Pure bacterial isolates from the inoculated seedlings were identified based on colony morphology on NA, pigment production on KB, and non-fluorescence of bacterial colonies.

On KB medium bacterial isolates were recovered from *B. humidicola* CIAT accession no.16888. These had a biochemical reaction similar to *B. glumae* reference isolate 3252-8. Isolated colonies on the KB medium closely resembled *B. glumae* colonies. They produced a non-fluorescent yellowish-green pigment.

Bacterial strains were initially stored on filter paper in darkness at  $-20^{\circ}\text{C}$  as described previously [28]. Strains were then purified by serial dilution with sterile distilled water and plated on King's B agar (KB) (20 g protease peptone, 1.5 g  $\text{KH}_2\text{PO}_4$ , 1.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 15 g Bacto-agar and 15ml glycerol). Plates were incubated at  $30^{\circ}\text{C}$  for 48 h. Bacterial colonies were examined for their morphological characteristics and compared to the type strain, *B. glumae* 3252-8. Single colonies were stored in microcentrifuge tubes at  $-20^{\circ}\text{C}$  in 50% glycerol for further characterization.

Strains of *Burkholderia* spp. from the American Type Culture Collection (ATCC) were used in this study and included: *B. glumae* (ATCC 33617), *B. gladioli* (ATCC 33664 and 19302), *B. plantarii* (ATCC 43733 and 51545), *B. multivorans* (ATCC BAA-247) and *B. cepacia* (ATCC 10856).

### Pathogenicity tests

The pathogenicity of 32 putative *B. glumae* strains was evaluated by inoculating leaves of the interspecific *Brachiaria* hybrid cv. 'Mulato II' (CIAT 36087). Before pathogenicity tests, bacterial strains were inoculated to KB, incubated at  $30^{\circ}\text{C}$  for 24 h, harvested with a glass rod and suspended in sterile distilled water. For inoculation, 0.1 ml of bacterial suspension ( $10^8$  CFU/ml) was applied by puncturing on the surface of leaf sheaths. Lesion lengths on the inoculated sheaths were recorded 7 d after inoculation. Three replicate tests were performed for each bacterial strain and bacteria-free suspensions were used as controls.

Koch's postulates were accomplished. First the bacterium was found in diseased but not healthy plants, secondly the bacterium was cultured from the diseased plants inoculated with the bacteria showing disease symptoms, and finally the bacterium was reisolated from the inoculated diseased plants and was identical to the original bacterium.

### Virulence studies

The virulence of *B. glumae* strains was evaluated by inoculating 10 *Brachiaria* genotypes from the closely-related cultivated species, *B. brizantha*, *B. decumbens*, and *B. ruziziensis*, and hybrid cultivars derived from interspecific crosses of these species. The *Brachiaria* genotypes included in these tests

included *B. brizantha* cv. 'Marandú' (CIAT 6294), *B. brizantha* cv. 'Toledo' (CIAT 16320), *B. brizantha* cv. 'Piata' (CIAT 16125), *B. decumbens* cv. 'Basilisk' (CIAT 606), *B. ruziziensis* (BRX 4402), *Brachiaria* interspecific hybrid CIAT 36062 and interspecific hybrid *Brachiaria* cultivars 'Mulato I' (CIAT 36061), Mulato II (CIAT 36087), 'Cayman' (BR02/1752) and 'Cobra' (BR02/1794) (Table 2). Plants from each genotype were prepared for virulence tests by detaching tillers (20 cm to 25 cm) from parental plants; tillers were then immersed for 5 min in a 1% solution of sodium hypochlorite. The tillers (clones) were then planted individually in PVC tubes (5.3 cm  $\times$  6.5 cm) containing 36 g of sterilized soil (nursery mixture of soil and sand in a 4:1 ratio). The PVC tubes and plants were arranged in a completely randomized experimental design with eight replicates and two negative controls per genotype. The transplants were grown in the greenhouse, watered every two days and fertilized one week before inoculation.

Forty-day-old clonal transplants were inoculated with 0.5 ml of a bacterial suspension ( $10^8$  CFU/ml). Negative controls were inoculated with sterile distilled water. Individual inoculated plants and controls were incubated in the greenhouse at  $32^{\circ}\text{C} \pm 3$ . The above-ground portion of each plant was enclosed in a 600 ml clear plastic water bottle. The bottle acted as a micro-chamber to create conditions of high relative humidity (80% to 100%). The bottles also physically isolated each plant, preventing leaf contact between adjacent plants [29]. Each plant was evaluated for the presence or absence of symptoms (+/-) and the percentage of the total area affected 10 days after inoculation. Virulence assessments were analyzed using SAS Software v. 9 of the SAS System for Unix (Cary, North Carolina, USA) [30] using the MIXED procedure and REML estimation method.

### Species-specific PCR with 16S rDNA primers

Bacterial strains (Table 1) were inoculated to KBA and incubated for 48 h at  $28^{\circ}\text{C}$ . Bacterial cells were collected from 1.5 ml of culture ( $10^8$  CFU/ml) by centrifugation at 11,000

**Table 2:** Host plants used to determine the virulence of *Burkholderia glumae* on *Brachiaria* genotypes.

Host plants	CIAT accession number <sup>a</sup>	n <sup>b</sup>	Percent disease leaf area <sup>c</sup>	Grouping
<i>B. brizantha</i> 'Marandú'	CIAT 6294	10	18.0	A
<i>Brachiaria</i> hybrid 'Cobra'	BR02/1794	10	18.0	A
<i>Brachiaria</i> hybrid 'Mulato II'	CIAT 36087	10	16.0	AB
<i>B. ruziziensis</i>	BRX 4402	10	15.5	AB
<i>Brachiaria</i> hybrid 'Mulato'	CIAT 36061	10	11.5	ABC
<i>B. brizantha</i> 'Piata'	CIAT 16125	10	11.5	ABC
<i>B. brizantha</i> 'Toledo'	CIAT 26110	10	10.5	BCD
<i>Brachiaria</i> hybrid 'Cayman'	BR02/1752	10	5.5	DE
<i>B. decumbens</i> 'Basilisk'	CIAT 606	10	5.0	DE
<i>Brachiaria</i> hybrid	CIAT 36062	10	0.0	E

<sup>a</sup>CIAT = International Center for Tropical Agriculture

<sup>b</sup>n = number of plants inoculated per genotype

<sup>c</sup>Mean difference between each group is significant at the 0.05 level; values with the same letter based on REGW multiple range test in a column do not differ significantly. Data expressed as a percentage.

rpm for 1 min and used for DNA isolation. Bacterial DNA was isolated using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI) following the manufacturer's instructions. The quality of DNA was checked on agarose gels and quantified using a Thermo Scientific NanoDrop 2000c UV-Vis spectrophotometer (NanoDrop, Wilmington, DE).

For identification of *B. glumae*, template DNA was used in PCR and amplified with 16S rDNA primers specific for *B. glumae*: 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-AAGGAGGTGATCCAGCC-3'), which were previously reported by Frank, et al. [31]. To identify pathogenic *B. glumae* strains, primers described by Sayler, et al. [32] were used in the PCR reactions: gluF (5'-ACGTTTCAGGGATRCCTGAGCAG-3') and gluR (5'-AGTCTGTCTCGCTCTCCCGA-3'). Each 25 µl PCR reaction contained the following: 1 µl each of forward and reverse primers (10 ng/µl), 12.5 µl Taq polymerase (GoTaq®, Promega) at 5 U/µl, 3 µl distilled water, and 3 µl bacterial DNA at 10 ng/µl. The reactions were carried out in a thermal cycler (PTC-100 Peltier Thermal Cycler; MJ Research, Inc.) using the following conditions: initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and final extension at 72 °C for 7 min. PCR products were resolved in 1.2% agarose gels in TAE buffer (1× Tris base, acetic acid and EDTA) and visualized with GelRed Nucleic Acid Gel Stain, 10,000 × (Life Technologies, India, Pvt. Ltd.).

### Sequence analysis of 16S rRNA

Before sequence analysis, a 1500-bp PCR fragment encoding 16S rRNA was obtained from *B. glumae* strains with primers 27 f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r (5'-AAGGAGGTGATCCAGCC-3') [31]. PCR products were purified with Polyethylene Glycol (PEG) as follows: PCR products were added to 1.5 mL of 20% PEG solution (10 g PEG 8000 and 7.3 g NaCl), mixed by vortexing, and incubated at ambient temperature for 15 min. The solution was centrifuged at 15,800 × *g* for 15 min at room temperature, the supernatant was discarded and 100 µl of 70% ethanol was added. This new solution was centrifuged at 15,800 × *g* for 2 min at ambient temperature, the supernatant was discarded, and the remaining pellet was incubated at 37 °C to dryness. The resulting DNA pellets were re-suspended in 20 µl of sterile distilled water.

Purified PCR products were sequenced at the DNA Facility located at Iowa State University, Office of Biotechnology. 16S rDNA sequences of *Burkholderia* spp. from the American Type Culture Collection (ATCC) were also included for comparative purposes. These included *B. glumae* ATCC 33617, (GenBank accession no. AMRF01000143.1), *B. gladioli* ATCC 19302 (accession no. AB190737) and 51545 (accession no. AB220914), *B. multivorans* ATCC BAA-247 (NZ ALIW01000369.1), *B. cepacia* ATCC 10856, *B. caryophyllii* ATCC 25418 (AB190670.1), *B. andropogonis* ATCC 19311 (AB190668.1) 16S rDNA sequences were also obtained from *Pantoea stewartii* ATCC 8199 (accession no. NR-044800.1), *Pseudomonas putida* KT2400 (NR-074596.1), *Pseudomonas marginalis* ATCC10844 (NR-112072.1), *Pseudomonas fluorescens* ATCC 17400 (NZ JENC01000059.1) and *Xanthomonas translucens* pv. *poae*. ATCC 33804 (MADN01000503.1). *B. glumae*

rDNA sequences from rice strains 3161-2 and 3193-5 and *B. gladioli* 3845s-5 (also from rice; accession no. JF 323581) were also analyzed.

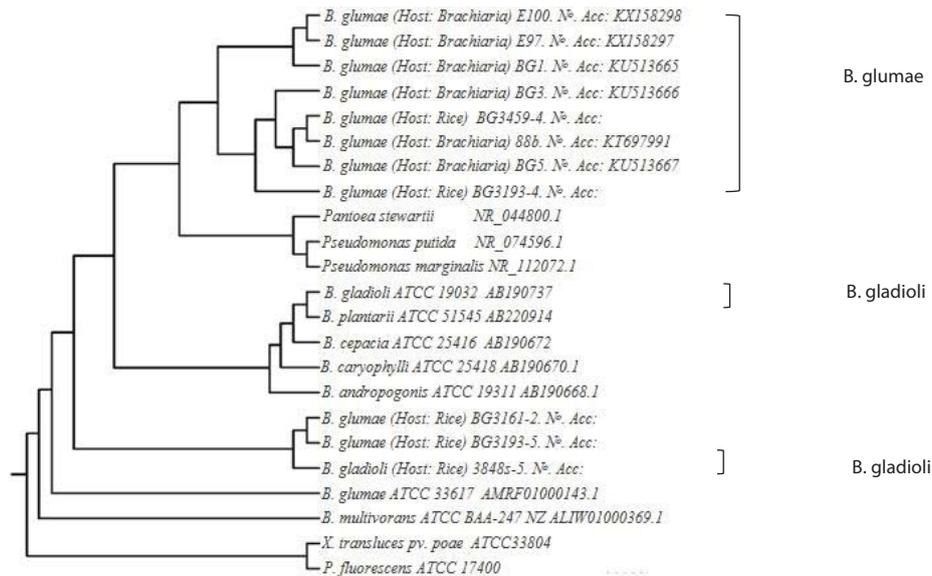
The sequences were initially aligned with Chromas PRO (Technelysium Pty Ltd.) and then compared with available sequences from the European Molecular Biology Library (EMBL) and GenBank using BLASTN. Cluster analysis was performed on individual sequences of the 23 strains of our bacterial collection. Strain ATCC33804 and ATCC17400 were used to root. Using unweighted pair-group method of averages (UPGMA). This was analyzed using NTSYS PC 2.2 program and a tree was generated

### Real-time PCR identification of bacterial strains

DNA was isolated for real-time PCR from *B. glumae* strain 3252-8 (type strain), 32 strains of *B. glumae* (this study), *B. gladioli* (rice strain no. 3845-5), *Pseudomonas putida*, *P. marginalis*, *Pantoea stewartii*, *P. aeruginosa* and *Xanthomonas campestris* pv. *graminis*. Bacterial cells (OD<sub>600</sub> = 0.5) from 1 mL of culture were centrifuged at 8600 × *g* for 3 min, and the pellet was used for DNA isolation as described previously [11]. The method used for DNA isolation was also used for direct PCR and sequence analysis. Primers for real-time PCR were synthesized based on the sequence of the Internal Transcribed Spacer (ITS) of *B. glumae*, and primers were used to previously detect this pathogen [11]. The following primers were used for real-time PCR reactions: forward primer, FP-BUR (5'-CAAGATGATTGCAACGCAAGTT-3'); reverse primer: RP-BUR (5'-TCGCTCTCCCGAAGAGAT GA-3'); and probe P-BUR (5'-TACGGCACA AATGCGAGAACTCAACCT-3'). The real-time PCR experiment was standardized as described previously [11]. Briefly, different probe concentrations (50 to 300 nM), primer concentrations (2.5, 5, 10, 15 and 20 pmole), and annealing and extension times (20, 30, 40 and 60 s) were tested using a uniform template concentration of *B. glumae* 3252-8 (type strain). The probe P-BUR (5'-TACGGCACA AATGCGAGAACTCAACCT-3') was used for real-time reactions. The TaqMan probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end as described previously [11].

Real-time PCR was performed using TaqMan universal PCR master mix and the MJ Research Thermal Cycler (PTC-100). The 30-µl reaction mixture consisted of 15 µl of 2 × master mix, 1 µl each of forward and reverse primers (5 pmol/µl), 1 µl of probe (100 nM) and varying amounts of DNA template and sterile water. PCR conditions consisted of a 10-min preincubation period with a 15-s denaturation at 95 °C leading to 40 cycles of annealing and extension at 60 °C for 20 s. In all experiments, the nontemplate DNA control was maintained as a negative control, and three replicates were performed for each sample.

The obtained sequences were initially aligned with the Chromas PRO program and subsequently compared with sequences available in EMBL/GenBank database using BLASTn.



**Figure 1:** E. Alvarez.

Phylogenetic tree constructed by UPGMA analysis of near-full-length 16S rDNA sequences from bacteria isolated from *Brachiaria* in Colombia (strains E100, E97, BG1, BG3, BG5, and 88b). Reference strains included *B. glumae* BG 3459-4, BG 3193-4, 3161-2, 3193-5, and ATCC 33617. 16S rDNA sequences of *Burkholderia* spp. from the American Type Culture Collection (ATCC) were also included for comparative purposes. These included *B. gladioli* ATCC 19302, (accession no. AB190737), *B. plantarii* 51545 (accession no. AB220914), *B. multivorans* ATCC BAA-247 (NZ ALIW01000369.1), *B. cepacia* ATCC 25416, *B. caryophylli* ATCC 25418 (AB190670.1), *B. andropogonis* ATCC 19311 (AB190668.1) 16S RNA sequences were also obtained from *Pantoea stewartii* ATCC 8199 (accession no. NR\_044800.1), *Pseudomonas putida* KT2400 (NR\_074596.1), *Pseudomonas marginalis* ATCC 10844 (NR\_112072.1), *Pseudomonas fluorescens* ATCC 17400 (NZ JENC01000059.1), and *Xanthomonas translucens* pv. ATCC 33804 (MADN01000503.1). Numbers after bacterial names are GenBank accession numbers.

The dendrogram was generated using the unweighted per-group method of averages (UPGMA) analysis of the DNA sequences. The NTSYS PC Version 2.2 program by pair-wise comparison was used, and the results demonstrated the overall similarities among strains (Figure 1).

## Results

*Brachiaria* spp. grown in CIAT fields exhibited bacterial blight symptoms (Figure 2A). Bacteria were isolated from diseased *Brachiaria* spp. on King's B medium and purified by serial dilution; single colonies were transferred to fresh medium. Pure cultures were stored in King's B broth (KBB). A total of 82 bacterial strains were isolated and purified on King's B agar medium. In this work, 32 strains (Table 1) were selected because they resembled *B. glumae* according to morphological characteristics (Gram-negative rods) and yellow pigmentation on KBA.

### Pathogenicity tests

Bacterial strains ( $n = 82$ ) from diseased *Brachiaria* were tested for pathogenicity on the interspecific hybrid *Brachiaria* cv. Mulato II. Koch's postulates were confirmed by re-isolating *B. glumae* from diseased sheaths showing blight symptoms. Thirty-nine bacterial strains were pathogenic and produced a yellow pigment on KBA; 32 of these strains (Table 1) were selected for further study because they resembled *B. glumae* according to morphological characteristics (Gram-negative rods) and pigmentation. Ten bacterial strains were nonpathogenic and did not produce a yellow pigment on KBA.



**Figure 2:** E. Alvarez.

Bacterial blight symptoms on *Brachiaria* spp. Typical field symptoms of bacterial blight in susceptible *Brachiaria* (left row). Healthy *Brachiaria* is shown on the right. B, Foliar symptoms of bacterial blight in *Brachiaria*. Chlorosis is evident along the midribs and flag leaf margins.

Pathogenicity tests revealed that nine (28.2%) of the 32 tested strains were highly pathogenic in the leaves of *Brachiaria* cv. CIAT 36087 Mulato II. The nine highly pathogenic strains induced symptoms similar to known strains of *B. glumae* and included E84, E85, E91, E92, E94, E97, E100, E103 and 88b. Negative controls were nonpathogenic. All pathogenicity experiments were repeated twice.

Our results indicate that *B. glumae* was the pathogen causing bacterial blight in Colombia.

### Virulence studies

*B. glumae* strain 88b exhibited virulence on *Brachiaria* hybrid cv. Cayman (BR02/1794), *B. brizantha* cv. Marandú (CIAT 6294),

*Brachiaria* hybrid cv. Mulato II (CIAT 36087), and *B. ruziziensis* BRX 4402 (Table 2). Symptoms included yellowing and chlorotic streaks in the flag leaf (Figure 2B). The percentage of leaf areas showing symptoms ranged from 15.5 to 18.0% (Table 3). Strain 88b infected all *Brachiaria* genotypes except *Brachiaria* interspecific hybrid CIAT 36062 (Table 2), which was identified as highly resistant.

### Identification of strains by PCR and sequencing

The 32 pathogenic bacterial strains were further analyzed using species-specific primers for *B. glumae* [32]. *B. glumae* strain 3252-8 was used as a positive control. Amplicons of the expected size (286 bp) were generated from six strains (Figure 3).

In the amplified region, 16S rDNA of DNA isolates from diseased *Brachiaria* leaves and commercial seed samples showed a 1500 bp molecular weight band, and this region was sequenced. Six bacterial strains belonging to *Burkholderia glumae* (GenBank accession numbers KT697991, KU513665, KU513666, KU513667, KX158297 and KX158298) were identified by PCR.

A total of 23 strains including other *Burkholderia* species and related species from *Brachiaria* and rice in Colombia were screened. Sequence analysis of the 16S rDNA genes from *B. glumae* strains (88b, BG1, BG3, BG5, E97, E100, BG 3193-4) three *B. gladioli* strains (*B. gladioli*, 3161-2, 3193-5) showed 99% sequence similarity with already published *B. glumae* sequence 16S rDNA and ITS sequences for strains of the bacterial species were submitted to GenBank (accession numbers KT697991, KU513665, KU513666, KU513667, KX150277, KX158298).

By DNA sequencing, the bacterial strains were identified as *Burkholderia glumae*, with a sequence homology of 99% when compared with reference strains (Table 3).

### Real-time PCR identification of bacterial strains

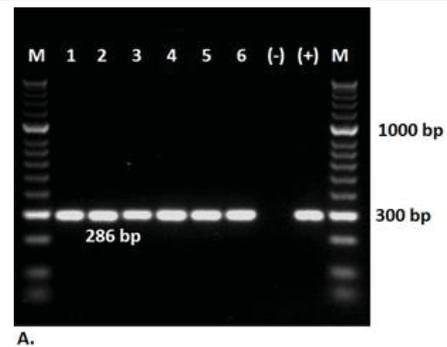
*B. glumae* primers FP-BUR/RP-BUR and the probe P-BUR were previously used in real-time PCR to specifically detect and quantify *B. glumae* [11]. Thirty amplification cycles were sufficient to obtain positive Ct values in the range of 11-16 cycles of amplification. Cycle threshold (Ct) values were compared with a standard curve to identify and determine bacterial species from tissue samples. The parameters described by Nandakumar, et al. [11] were used to develop the standard curve. Concerning the control (no template added), fluorescence was either absent or marginal until the last amplification cycle.

The specificity of primers and probe were evaluated in real-time PCR. DNA isolated from the reference strain *B. glumae* BG 3193-4 and 88b, BG1, BG3, BG5, E97, and E100 from *Brachiaria*, *B. glumae* strain from Colombia, and others were tested. Amplification showed that *Brachiaria* strains had the same amplification profile as the control strain BG 3193-4 from rice no signal was detected for *B. gladioli* and nontemplate control after 30 cycles

**Table 3:** Identification of *Burkholderia* strains (in this study) using comparisons of sequences of 16S ribosomal DNA regions with those in the GenBank database.

Strain code	Accession Number (This study)	Name, similarity % <sup>a</sup>	Reference accession number (Gen Bank database)
88b	KT697991	<i>B. glumae</i> 99	KF995708.1
BG5	KU513665	<i>B. glumae</i> 99	KF995706.1
BG1	KU513666	<i>B. glumae</i> 99	CP009435.1
BG3	KU513667	<i>B. glumae</i> 98	KF995706.1
E97	KX158297	<i>B. glumae</i> 99	JQ994134.1
E100	KX158298	<i>B. glumae</i> 99	KX158297.1

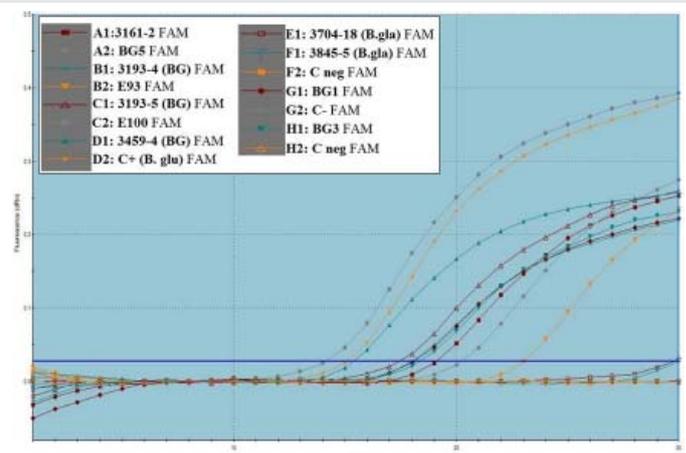
<sup>a</sup>Percent similarity of sequenced 16S ribosomal DNA region with GenBank database accessions



A.

**Figure 3:** E. Alvarez.

PCR analysis of bacterial strains with primers gluF/gluR, which are specific for the detection of *B. glumae*. Lanes: 1, strain BG1; 2, BG3; 3, BG5; 4, E93; 5, E97; 6, E100; 7, no template (negative control); 8, *B. glumae* type strain 3252-8 (positive control). *Burkholderia glumae* isolates were obtained from *Brachiaria humidicola* CIAT 16888. M, Molecular weight DNA marker.



**Figure 4:** E. Alvarez.

Real-time PCR assay with *B. glumae* primers. Relative fluorescence ( $\Delta R_n$ ) of the probe is shown by cycle amplification of bacterial strains with *B. glumae* specific primers F-BUR/P-BUR and probe P-BUR. *B. glumae* strains included 3161-2, BG5, 3193-4, E93, 3193-5, E100, 3459-4, 3252-8 (type strain, positive control), BG1, and BG3; *Burkholderia gladioli* strains 3704-18 and 3845-5 were also included. Negative controls consisted of reactions with no DNA template.

Twenty-eight strains were positively amplified in Figure 4.

These virulent, yellow-pigmented strains were previously amplified and sequenced by conventional PCR DNA sequencing and were submitted to GenBank with the following accession

numbers 88b: KT697991, BG1: KU513665, BG3: KU513666, BG5: KU513667, E97: KX158297 and E100: KX158298.

## Discussion

Bacterial blight symptoms have been documented in *Brachiaria*, but the causal pathogen was previously identified as a species of *Xanthomonas* [33].

This study confirmed that *Burkholderia glumae* isolated from *Brachiaria humidicola* CIAT 16888, CIAT 16889, CIAT 16890, CIAT 16891, *Brachiaria* hybrid CIAT 36061 cv. Mulato, and *Brachiaria* hybrid CIAT 36087 cv. Mulato II at CIAT (Palmira, Colombia) was pathogenic on several species of *Brachiaria*.

*Burkholderia glumae* causes both rice seedling and grain rot in the field. The bacterium produces a host-non-specific phytotoxin, toxoflavin, which is a major virulence factor [34]. Toxoflavin is a phytotoxin with a broad host range, which is a key virulence factor in bacterial grain rot. We isolated bacterial strains from leaves of *Brachiaria* in Colombia in 2009 and 2016. Initial observations of bacterial strains in the KBA medium and pathogenicity tests (phenotypic characteristics) suggested that *B. glumae* species might be involved, considering that the bacteria produce toxoflavin, resulting in yellow pigmentation on the KBA medium. By PCR and sequencing, we could determine the identity of the pathogenic strains of *B. glumae* with a percentage of homology of 99%.

In addition, molecular techniques have been designed for the identification of *B. glumae* based on specific DNA sequences in the spacer region between the genes coding for the 16S rDNA and 23S rDNA [35]. PCR has been used to identify pathogens causing new diseases and even to differentiate species within the same genus [34,36,37]. Genotypic differences between *B. glumae* and *B. gladioli* strains were identified [18]. PCR has also been used as a method for identifying several *Burkholderia* species from soil, water, and infected plants [22,36–38,39]. A real-time PCR method that is effective in detecting and identifying *B. glumae* in seed lots and plant tissue has been used [32]. The real-time PCR assay described here detects the pathogen in leaves, eliminating the need for visualization of PCR products on an agarose gel. Real-time PCR also reduces assay time from 6h to 2h and real-time data will be available. It was the most sensitive and accurate method evaluated for identifying the pathogen.

In our pathogenicity test, the strains showed differential reactions to the pathogen. The strain 88b (*B. glumae*) was more virulent in genotypes *B. ruziziensis* (BRX 4402), *B. brizantha* ‘Marandú’ (CIAT 6294), *Brachiaria* hybrid ‘Mulato II’ (CIAT 36087), and *Brachiaria* hybrid BRO2/1794 (Table 3).

The methodology for *in vitro* inoculation of leaf blades to assess pathogenicity and the microchambers methodology used in evaluating the virulence of *B. glumae* in genotypes of *Brachiaria* spp. were shown to be reliable and reproducible, allowing us to obtain reliable results concerning pathogenicity and virulence.

*Burkholderia glumae* was pathogenic on *Brachiaria*. Phytopathogenic species of *Burkholderia* synthesize a variety of toxins such as toxoflavin, ferverulin, and tropolone [34]. Toxoflavin is necessary for pathogenicity by *Burkholderia* causing both seedling disease and grain rot, for example, in rice. Several virulence factors are reported in *B. glumae* including catalase, flagellar biogenesis, a type III secretion system, and the main mediator of damage, toxoflavin.

The damage caused by *B. glumae* depends on its ability to multiply abundantly and generate high-density toxoflavin production. This causes H<sub>2</sub>O<sub>2</sub> generation by tissue damage and obstruction of the vascular bundles on the ground, preventing the arrival of monosaccharides and disaccharides essential for the formation of foliage [10]. This obstruction also induces symptoms of chlorosis, which is responsible for bacterial wilt.

The range of interactions between these genera of bacteria and their hosts is complex and diverse. The interactions of some species seem restricted to one type of host, whereas others have a much wider host range for *B. glumae*. The type of interaction may be that of a pathogen, but it can also be symbiotic or both [11,17,40–42]. It is proposed that the pathogen *B. glumae* is the causal agent of bacterial blight in *Brachiaria*, showing different degrees of aggressiveness and virulence. We will therefore need to evaluate additional *Brachiaria* spp. genotypes against bacterial strains of *B. glumae*.

It has been established that the bacteria are found in the phylloplanes of rice plants [43,44], stored rice seeds [45], weeds in the field, and tissues of rice crops that were placed on the ground [46]. The genus *Brachiaria* helps improve soil compaction because it has roots that can penetrate the compacted subsoil. Sustainable strategies for managing bacterial blight in rice have been described [47]. Crop rotation alters the biological, chemical, and physical properties of soils, and involves planting different crops in sequence on the ground. Farmers should alternate crops to break the life cycle of pests and diseases and use various methods of control. The affected crop or crops need to be removed during these alternate periods to break the life cycle of diseases and pests that want to remain, which is an important point considering the rotation of crops involving both the host (*Brachiaria*) and the pathogen (*B. glumae*) [48]. To combat diseases, this practice is effective if the crops in the sequence are not the same and the host pathogens do not have long-term survival mechanisms in the absence of the host. Comparative genome analysis of rice-pathogenic *Burkholderia* provides insight into the capacity to adapt to different hosts [49]. Evaluation of major rice cultivars for resistance to bacterial seedling rot caused by *B. glumae* has been conducted and identification of Japanese standard cultivars for resistance assessments [50].

Screening for disease resistance using stem injection inoculation of *Brachiaria* grown in the greenhouse allows us to identify resistance to *B. glumae*. This greenhouse assay could be useful to breeders for developing cultivars resistant to bacterial blight.



This study established the genetic similarity of *B. glumae* strains from Colombia and the first characterization and also presents the implementation of a real-time PCR detection assay for *B. glumae* in *Brachiaria* leaves (Table 4).

## Funding

International Center for Tropical Agriculture. Alvarez, E., Latorre, M. 2023. Bacterial Blight of *Brachiaria* caused by *Burkholderia glumae* in Colombia.

**Table 4:** Analysis of variance of the percentage of leaf area affected with *Burkholderia glumae* inoculated on *Brachiaria* genotypes for the virulence study.

Source of variation	DF	Sum of squares	Mean square	F value	Pr > F
rep	9	320.25	35.58	1.59	0.1320
Treatment (Genotype)	9	3310.25	367.80	16.44	<0.001

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