

Endophyte-mediated biocontrol reduces the pathogenicity of *Burkholderia glumae* in rice seedlings (*Oryza sativa* L.)

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ABSTRACT

Burkholderia glumae is the pathogen responsible for causing bacterial panicle blight, a disease that affects rice (*Oryza sativa*) and significantly impacts crop production and yield efficiency. Current control methods are mainly based on agrochemicals and are less effective against the emergence of new strains. It is, therefore, necessary to find alternative methods to reduce the impact of the pathogen in the field. This study aims to evaluate the possible biocontrol effect of endophytic bacteria isolated from rice against symptoms caused by the pathogen *Burkholderia glumae* in rice seedlings. Initial results from this research led to the identification of 16 endophytic isolates with characteristic *in vitro* PGPB. In the test on seedlings, the endophytic improved the vigor index values. Subsequently, three strains of *B. glumae* were assessed for their pathogenicity, involving the determination of severity levels and their impact on seedling physiology. Following this, the capacity of endophytic bacteria to ameliorate effects of the disease on rice seedling physiology was evaluated. The results of the study revealed that the endophytic isolates Ory09 and Ory59 exhibited the ability to reduce the severity of the disease, as well as inhibit the coleoptile and radicle impairment caused by *B. glumae*. The data indicated that these two isolates did not affect the vigor index concerning healthy controls.

1. Introduction

Burkholderia glumae, identified by Kurita & Tables i and is the causative agent responsible for bacterial panicle blight in rice (*Oryza sativa*). This disease is one of the most economically important diseases in the world due to the significant crop losses it causes. Field losses are estimated to be between 60 % and 75 % of production [1]. Managing bacterial blight in the field has proven challenging primarily because *B. glumae* thrives under conditions conducive to rice growth [2,3], and early-stage detection has been problematic [4] due to the absence of visible symptoms, which only manifest during the flowering stage [5]. One of the methods used in the field is the selection of varieties through genetic improvement to obtain disease-resistant rice lines. However, the developed varieties have a low resistance to *B. glumae*, making new rice lines a short-term control method. In addition, the genotypic characteristics of these varieties do not maintain optimal production

characteristics in terms of germination, vigor, robustness, tillering potential, and yield, making them unattractive at a commercial level [6]. The symptoms of the disease occur in two phases: the first, the vegetative phase, is characterized by the presence of blight on stems and leaves, rot, chlorosis, and complete maceration of the beetle and plumule [7]; and the second, the flowering phase, presents symptoms mainly in the panicle, with straw-colored spikelets, discoloration of the grain, rot, and venting [3]. In addition, *B. glumae* can be present in the rice plant from germination without presenting symptoms in the first phenological phases of the crop, developing symptoms under favorable flowering conditions, a characteristic that makes it difficult to establish effective control methods for this disease [2,8–10]. These encompass a range of effects including reduced grain weight, panicle blight, seedling blight, compromised seed germination, spikelet sterility and inhibition of grain filling [10,11]. Current biocontrol strategies employed against *B. glumae* have proven ineffective and have given rise to additional

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issues, notably of an environmental nature [12]. For instance, oxolinic acid, a quinoline derivative, is utilized as an agrochemical to mitigate disease severity in crop fields by inhibiting bacterial growth through DNA synthesis suppression [13]. However, excessive application in agricultural contexts can lead to the development of new pathogenic strains and the presence of trace residues in soil, contributing to environmental contamination [13,14]. Other control approaches involve the use of resistant cultivars developed through conventional genetic techniques. Regrettably, this method has proven ineffective due to the eventual loss of disease tolerance [3,11].

Therefore, research efforts need to be directed toward identifying effective control alternatives, including using beneficial microorganisms to mitigate the impact of *B. glumae* on crops. Recent studies focusing on biocontrol tactics have proposed endophytic bacteria as a promising avenue for pathogen management within agricultural fields [15–17]. Unlike other beneficial microorganisms, endophytic bacteria possess the unique ability to inhabit internal plant tissues, forging robust interactions with the host plant and directly influencing its metabolic processes [15,18]. Numerous investigations have highlighted the involvement of endophytic bacteria in mechanisms that promote plant growth [19–21]. These mechanisms encompass nutrient provision [22] as well as defense against pathogens [16]. For example, recent studies on six rice varieties identified endophytic bacterial communities belonging to the phyla Proteobacteria and Firmicutes. 32 isolates were identified with the potential to solubilize phosphate and produce AIA and siderophores. The same bacteria showed antagonism against bacterial and fungal pathogens. Finally, the production of antibacterial metabolites such as surfactin and antifungal metabolites such as iturin D and bacillomycin was detected [20]. Kalboush et al. [23] developed a nano formula with nano natural charcoal and alginate to immobilize endophytic bacteria and evaluate their ability to promote growth and induce defense mechanisms against *Rhizoctonia solani*. Data from this study on adult rice plants showed higher growth percentages in the assessed parameters compared to chemical control. In addition, there was a greater presence of enzymes and genes involved in the plant defense response. In the context of pathogen defense, endophytic bacteria exert their influence either indirectly through the production of antimicrobial compounds [24] or through indirect means such as the induction of systemic resistance (ISR) [25].

Research on the biocontrol of *B. glumae* has remained relatively limited up to the present time. Certain publications have indicated that endophytic bacteria can inhibit the colonization of the pathogen in plant tissues in germinated seeds and seedlings older than 10 days [26,27]. Other includes research by the National University's Institute of Biotechnology (IBUN), which isolated endophytic and rhizospheric bacteria capable of reducing *B. glumae* symptoms by 62 % in experimental fields. Among the bacterial species studied, *Bacillus velezensis* stood out for its ability to reduce *B. glumae* populations and promote plant growth [26,28]. Another of the most representative studies is on *Streptomyces* for the biocontrol of *B. glumae*. In these studies, the species *Streptomyces corchorusii* stands out, with the potential to reduce the effects of *B.g lumae* on seedlings grown in greenhouses by 50 %. Studies carried out with these strains using formulations showed the stability of the biocontrol bacteria and their ability to reduce pathogen symptoms and increase plant growth by 300 % compared to the control [29,30].

The underlying mechanism through which endophytic bacteria bolster the defense system of host is termed the induced systemic response (ISR) [25]. Through ISR, endophytic bacteria can modulate molecular signals, placing the plant in a systemic state of alertness. This heightened state enhances the defense response during the infection process and fortifies the plant against future attacks [25,31,32]. Consequently, endophyte-mediated ISR could serve as an effective biocontrol alternative, countering the effects of *B. glumae*, which represents the primary source of disease infection [11]. This study aimed to assess the potential of endophytic bacteria in mitigating *B. glumae* pathogenicity in both susceptible and tolerant rice cultivars. To achieve

this objective, the pathogenic effects of three strains of *B. glumae* on seed germination were characterized and the protective capacity of growth-promoting endophytic bacteria for the biocontrol of *B. glumae* was evaluated.

2. Material and methods

2.1. Plant material

The 45-day-old rice plants were harvested at the end of the vegetative phase, characterized by active tillering, growing to a height, and leafing out. Two rice varieties, F67 and F68, were used to collect plant tissues and seeds required for each test. Seeds were obtained, from the germplasm bank of the Federación Nacional de Arroceros de Colombia (FEDEARROZ). Throughout the study, the seeds were stored, in the dark at 8 °C. Cultivars F67 and F68 have been selected as the preferred seed for sowing because of their fast initial growth and milling quality, but studies report susceptibility to different strains of *B. glumae* [33,34]

2.2. Isolation of endophytic bacteria

For endophyte isolation, 1 g of each plant tissue (roots, stems, leaves, and panicles) was collected. The seeds were subjected to disinfection by an initial wash with phosphate buffer, followed by washes with sodium hypochlorite, Tween 20, and 70 % alcohol, and finally, a phosphate buffer wash and several rinsing cycles with sterile distilled water [35] Disinfection was confirmed by seeding a tissue sample and an aliquot of the liquid in which the tissues were suspended in an R2A medium. The tissues were shaken, immersed in peptone water, and incubated at 30 °C for 2 h with constant agitation. Plate counting and selection of colonies were done on TSA medium based on their morphological characteristics.

2.3. Test to analyze the pathogenicity of *B. glumae* in rice seedlings

Three *B. glumae* strains (33C, 43A, and 4026–1) were sourced from the Institute of Biotechnology at the National University (IBUN) located in Colombia. These strains have been previously validated as virulent [36]. To maintain their viability, each strain was temporarily preserved in a 20 % glycerol solution at –80 °C. Using a calibration curve to 600 nm OD, four different concentrations were determined, ranging from 1×10^5 to 1×10^8 colony-forming units per milliliter (cfu/mL). The pathogenicity test was carried out on seeds of varieties F68 and F67, which were challenged with *B. glumae*. The test consisted of 24 sets of 80 seeds immersed in 100 ml of *B. glumae* inoculum and incubated at 30 °C for 24 h with shaking at 110 rpm. The seeds were transplanted into aluminum pots on absorbent paper moistened and incubated in a germination chamber at 30 °C for four days in the dark. A total of 8 pots were used for each treatment, with 10 seeds per pot. This test was repeated three times. The pathogenicity test was assessed by the severity of infection using a severity scale: 1 = Healthy seedlings, 2 = Healthy seedlings with loss of root vigor compared to the control, 3 = Development of aerial part with less than 50 % on the surface, 4 = Development of aerial part and more than 5 % discoloration, 5 = Development of aerial part and growth to 1 cm height, 6 = Coleoptile and plumule macerate without plant development [37]. The degree of infection was determined to assess the protective capacity of the endophytes in tests described below. The DI was calculated using the equation [38]:

$$DI = \sum (\text{Number of seeds in each severity level} \times \text{level}) / \text{Total number of seeds.}$$

In addition, the vigor index was calculated using the same germination percentage for all treatments according to the formula described by Anupama et al., [39].

2.4. *In vitro* evaluation of growth promotion activities of endophytic bacteria

Four *in vitro* biochemical assays were analyzed using the following referenced methods to determine the potential for endophytic growth promotion: Nitrogen-fixing activity [40], ACC deaminase enzyme [41], phosphate solubilization capability [42], and siderophore production using CAS medium [43].

Twenty-four endophytic bacterial isolates with positive results were tested for their growth-promoting ability in a greenhouse environment. Disinfected rice seeds from each variety were subjected to incubation in a bacterial suspension to 1×10^8 ufc/mL for 9 h. Sterile distilled water was utilized as the control treatment. After incubation, the seeds were transferred to aluminum pots ($48 \times 114 \times 143$ mm) and grown in a germination chamber at 28 °C for 4 days in the dark. The germinated sprouts were transplanted into pots (160 mm \times 200 mm) at the rate of one seed per pot. Each pot contained 2.5 Kg of autoclaved soil, infused with a 200 ml solution of bacteria. Greenhouse conditions were 30 °C, 70–80 % relative humidity, and 12.6 h photoperiod for 45 days. The experiment was replicated twice.

2.5. Evaluating the protective effect of endophytic bacteria in shoots against *B. glumae*

A total of nineteen treatment groups were established: sixteen treatments using a pre-inoculation with selected endophytic isolates rotated with the name Ory + numerical code, and then with *B. glumae*, one control challenged with only the pathogen, and one healthy control. Seeds from F68 and F67 rice varieties were subjected to challenge inoculation by immersing 18 sets, each containing 50 seeds, into an endophyte suspension at 10×10^8 ufc/mL in 5 % (vol/vol) peptone water. After this, the dried seeds underwent another challenge inoculation, this time utilizing a suspension of virulent *B. glumae* 43A at 10×10^5 ufc/mL in 5 % peptone water. The healthy controls were treated with sterile distilled water. The seeds were transplanted into aluminum pots containing absorbent paper and incubated at 30 °C and 80 % relative humidity for 5 days. Each treatment group comprised 5 pots, each containing 10 seeds. The results were obtained by analyzing the biometric parameters after five days of growth. The germination inhibition rate was also determined using the following equation [44]:

$$CIR (RIR) = \frac{LC (LR)_{control} - LC(LR)_{treatment}}{LC (LR)_{del control}} \times 100$$

Where: The coleoptile inhibition rate (CIR) and the radicle inhibition rate (RIR) are two parameters that determine the effect on the longitudinal growth of the coleoptile and the radicle formation concerning the healthy uninoculated control.

2.6. Molecular identification of endophytic bacteria

DNA extraction was performed according to the protocol described by Green et al. (2012). For the polymerase chain reaction, specific primers for the bacterial orders (α -proteobacteria, β -proteobacteria, γ -proteobacteria, and Firmicutes) were used that amplify the gene of the

16s rRNA region (Table 1). Positive controls including *Burkholderia cepacia*, *Escherichia coli*, and *Bacillus subtilis* strains were used for each primer. The amplified samples were sent to the laboratory of MacroGen Korea for sequencing. The resulting sequences have been compared with those previously reported in the Japanese DNA Bank (<http://blast.ddbj.nig.ac.jp/>). Base alignment was performed using Geneious Prime, Clustal W, and MEGA 11 software (Tamura et al., 2007).

2.7. Statistical analysis

Results meeting the criteria of normality and homoscedasticity were subjected to variance analysis with Tukey's correction at a significance level of 95 %. For nonparametric data, the Kruskal-Wallis test and the Dwass Steel Critchlow Fligner (DSCF) test were applied, with a p-value ≤ 0.05 . The statistical analysis was performed using Jamovi version 1.6.23 [47] and SPSS [48].

3. Results

3.1. Pathogenic effect of *B. glumae* on the vigor index of infected seeds

The results of the pathogenicity test using three strains of *B. glumae* and five different inoculum concentrations initially showed that strain 4026-1 did not affect seed growth on cultivar F68. However, strains 33C and 43A significantly reduced the vigor index, leading to decreased plant growth and development when compared to the control group. Consequently, strain 43A exhibited a notable negative effect on the vigor index, with average values approximately 87 % lower than those of the control group (Fig. 1). Regarding inoculum concentrations, no significant differences were observed among them, as they exhibited similar effects on seed growth.

For the F67 cultivar, strains 33C and 4026-1 yielded similar index values, resulting in a 58 % reduction in the vigor index. In contrast, the 43A cultivar displayed a 67 % reduction, with the lowest values recorded at a concentration of 108 cfu/ml-1. Both seed varieties displayed a high level of infection (degree infection 4.0). Symptoms were less severe in strains 33C and 4026-1 compared to strain 43A. In F68 variety seeds, symptoms included the presence of spots on over 50 % of the shoot, reduced radicle growth, and a significant negative impact on the coleoptile (Fig. 2). Conversely, in F67 variety seeds, symptoms were characterized by spots on less than 50 % of the plumule and brittle, hairless roots. Statistical analysis of F67 and F68 variety seeds treated with strain 43A revealed significant differences in symptom severity, making it the most aggressive strain among those evaluated in this study.

3.2. Selection of endophytes based on plant growth-promoting traits

A total of one hundred and fifty morphotypes were isolated from various plant tissues, including roots, stems, leaves and panicles. Among these morphotypes, positive isolates were selected for further evaluation of their *in vitro* growth-promoting capabilities. The results revealed that 41 isolates exhibited positive responses in at least one *in vitro* growth promotion test (Table 2). Out of the endophytic isolates, 25 demonstrated the ability to form solubilization halos on NBRIP medium after a five-day incubation period. Notably, the most extensive solubilization

Table 1
Specific primers of 16s rRNA gene to identify bacterial orders (α -proteobacteria, β -proteobacteria, γ -proteobacteria, and Firmicutes).

Primer	Sequences (5' – 3')	Reference
F203 α	CCGCATAGCCCTACGGGGAAAGTTTATCGCACAAAGCGGTGGATGA	[45]
F948 β^2	CGCACAAAGCGGTGGATGA	[45]
FD2 γ	AGAGTTTGATCATGGCTCAG	[46]
BLS342F	CAGCAGTAGGGAATCITC	
R1492 $\alpha\beta$	TACGG (C/T)TACCTTGTTCGACTT	[46]
RP1 γ	ACGGTTACCTTGTTCGCTT	[46]
R1392F	ACGGCGGTGTGTACA	

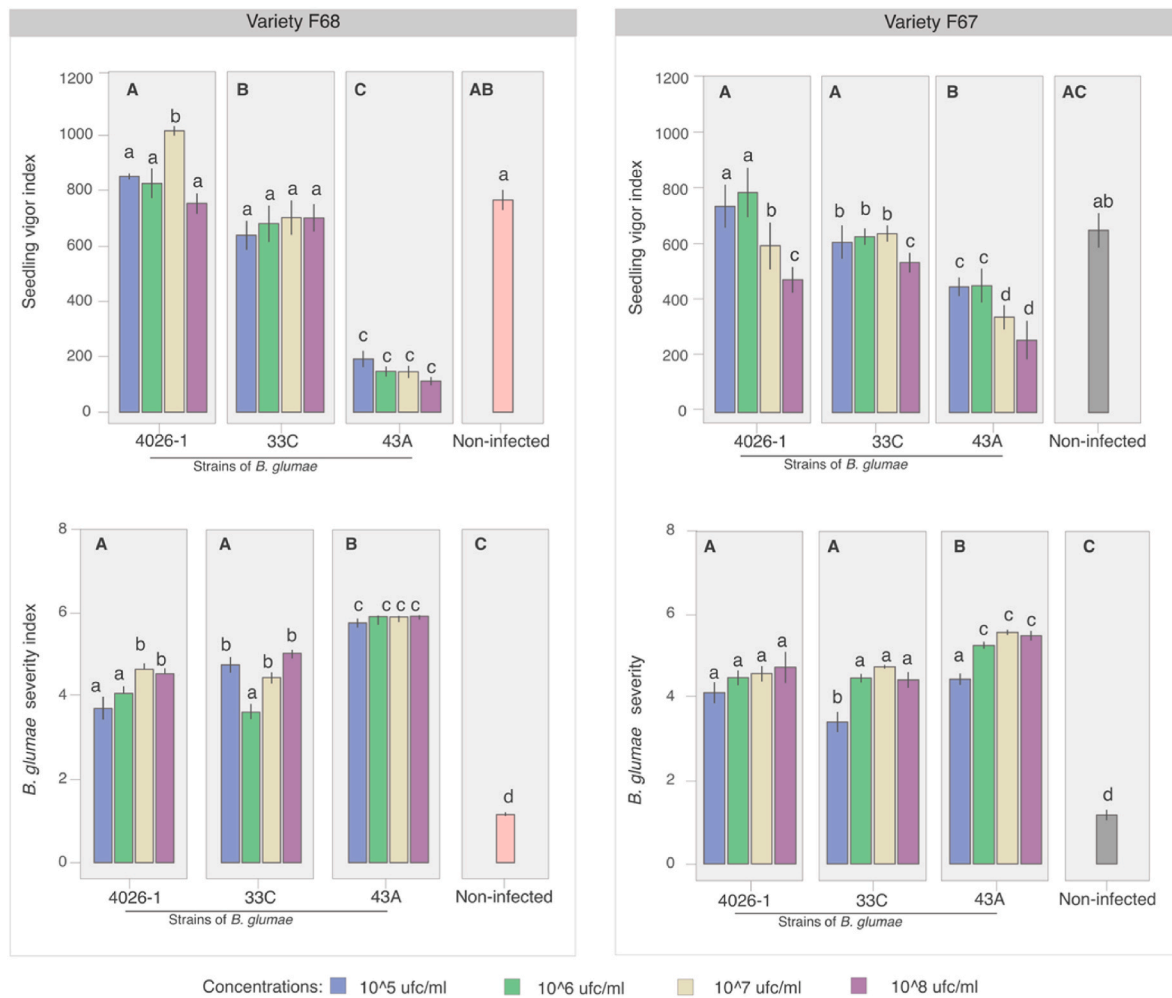


Fig. 1. Impact of three strains of *Burkholderia glumae* on the vigor index (top) and severity level (bottom) of the Fedearroz 68 (left) and Fedearroz 67 (right) rice varieties. Statistically significant are denoted by distinct lowercase letters and differences between strains are indicated by capital letters, at a p-value ≤ 0.05 .



Fig. 2. Symptoms observed in the various treatments involving the inoculation of rice seeds with different *Burkholderia glumae* strains in Fedearroz 67 and Fedearroz 68. The statistically significant distinctions between varieties concerning the severity level values for each. Different letters indicated statistical differences at $p \leq 0.05$.

halos were observed in isolates Ory28 and Ory59, with solubilization indices of 3.4 and 3.35, respectively. Furthermore, twenty-four of the isolates exhibited potential for siderophore production, with the highest siderophore production index values recorded for Ory55 (7.36) and Ory20 (5.67). Among the endophytic isolates, 16 were found to produce ACC deaminase, while 18 tested positive for ammonia production.

Ory58 was the only one that exhibit all plant growth-promoting characteristics tested. Recently, multifunctional microorganisms have become the focus of biotechnological studies due to their benefits in the agricultural sector. These microorganisms such as Ory58 can exert various plant growth-promoting activities, making them attractive for formulation biofertilizers to help reduce agrochemical inputs in crop

Table 2
Characteristics of growth promotion exhibited by endophytic bacteria.

Isolates	Trials PGP				F67		F68	
	FN	ACC	SP	PS	Stem dry weight (mg)	Root dry weight (mg)	Stem dry weight (mg)	Root dry weight (mg)
Ory2	-	+	1,23	3,32*	0,69	2,5	0,69	3,1
Ory5	-	+	1,68	1,43	0,67	3,1	0,68	2,3
Ory9	+	+	-	2,23	0,59	3,7*	0,72*	4,2*
Ory15	-	+	2,00	2,39	0,89	2,9	0,48	2,4
Ory16	-	-	2,82*	1,74	0,62	2,3	0,77*	3,4
Ory17	-	-	2,32	2,21	0,56	2,1	0,68*	2,4
Ory20	+	-	1,50	5,67*	0,64	2,3	0,61*	2,3
Ory22	-	-	2,22	1,46	0,70	2,2	0,56	2,4
Ory24	+	+	-	2,44*	0,87	3,0*	0,78*	3,2*
Ory27	-	-	1,33	2,88	0,55	1,8	0,65*	3,0
Ory28	-	-	3,40*	2,46	0,83*	3,5*	0,63*	3,4*
Ory29	-	-	-	-	0,59	2,5	0,71	4,4
Ory31	-	-	-	-	0,80	2,6	0,62*	2,6
Ory33	-	+	1,33	1,89	0,76*	1,9	0,68	3,5
Ory39	+	+	-	1,27	0,81*	3,0	0,51	2,2
Ory44	-	-	2,75	2,51	1,02*	3,0	0,61*	2,8
Ory45	-	-	2,23	1,31	0,79	3,7*	0,80*	3,9
Ory48	-	-	1,80	1,78	0,63	3,4*	0,73*	3,3*
Ory52	-	+	2,51	2,58	0,64	2,5	0,56	2,9
Ory54	+	-	-	-	0,73	2,1	0,77*	3,5*
Ory55	-	-	1,49	7,36*	0,80	3,7	0,71*	3,2*
Ory58	+	+	1,23	1,38	0,74	4,9*	0,84*	6,0*
Ory59	-	-	3,35*	1,54	0,53	2,1	0,58	2,7
Ory60	+	-	-	-	0,71	2,8	0,52	3,4
Control					0,49	1,9	0,39	2,0

FN - Nitrogen fixation; ACC - ACC deaminase; SP - Phosphate solubilization; PS - Siderophore production. *significant differences compared to the control treatment, $p \leq 0.05$.

fields.

3.3. Plant growth promotion activity of bacterial isolates *In vitro*

Plant growth-promoting capabilities of selected strains were assessed 45 days after pre-inoculation in the greenhouse. A total of 24 endophyte isolates were chosen based on their performance *in vitro* tests. Five days after the endophyte inoculation, significant effects on the root and stem dry weights of the seeds were observed compared to the control treatment (Table 2).

In the case of F68 seeds, the root dry weight exhibited a significant increase in 56 % of the tested isolates, whereas stem dry weight increased significantly in only 28 % of the isolates. Notably, isolate Ory58 displayed the most significant growth promotion, with a two-fold increase over the control treatment (0.85 mg for stems and 0.8 mg for roots compared to 0.38 mg and 0.2 mg, respectively, in the control). Additionally, isolates Ory33, Ory28, Ory54, Ory45 and Ory29 had a substantial impact on overall biomass, as measured by plant dry weight.

In the case of F67 seeds, only six endophytes exhibited a significant increase in growth promotion. Ory44 displayed a 2-fold increase in stem dry weight (1.02 mg), while Ory55 significantly enhanced root dry weight (4.9 mg) compared to the control treatment (1.9 mg). Among the isolates tested in F67, only Ory28 showed a lower but still significant increase in biomass (0.85 mg for stems and 0.8 mg for roots). These results suggest that the tested isolates demonstrated growth-promoting activity in plants irrespective of their genotype. Notably, isolates with the lowest bacterial activity were observed in the F67 cultivar, while those with the highest activity were found in the F68 cultivar 45 days after inoculation.

3.4. Protective effect of endophytic bacteria on seedlings against *B. glumae*

The ability of endophytic bacteria to reduce disease symptoms caused by *B. glumae* in rice seeds was assessed using three parameters: coleoptile inhibition rate (CIR), radicle inhibition rate (RIR), and degree infection (DI). Significant differences ($p < 0.05$) in CIR, RIR, and DI were

observed between the biocontrol treatments and the diseased controls for seedlings of both cultivars F68 and F67 (Fig. 3).

In the case of F67, three isolates significantly reduced coleoptile inhibition caused by the pathogen. Among these isolates, Ory28 and Ory09 demonstrated the highest average reductions in CIR, with values of 68 % and 60 %, respectively. The remaining isolates exhibited a percentage inhibition of less than 40 %. Additionally, among all the tested isolates, only Ory58 exhibited a 66 % reduction in the pathogenic effect, as evaluated by RIR on the root. The other isolates showed a range of RIR reduction between 40 % and 50 %, with Ory29 and Ory09 being notable. For the DI of F67 seeds, two groups of isolates were identified: the first group reduced the degree of infection compared to the diseased control, while the second group increased the DI value, as well as CIR and RIR values, surpassing those observed in the diseased control. Ory22, Ory29, Ory09 and Ory59 displayed the potential to mitigate the adverse physiological effects induced by *B. glumae*, with severity reduction ranging from 67 % to 51 %. In contrast, Ory54 exhibited higher disease severity values by 13 % compared to the infected control.

Similar results were obtained for F68 seeds in terms of biocontrol. In terms of CIR reduction, isolate Ory02 displayed a high capacity to reduce the pathogenic effect on the coleoptile, with a reduction rate of 68 % compared to the control treatment. In RIR, the percentage reduction in inhibition was 56.5 % in the treatments inoculated with Ory22. Isolates Ory55, Ory59 and Ory09 significantly reduced the impact of *B. glumae* by 40–50 % in both CIR and RIR. Furthermore, Ory09 was the most effective among the endophytic isolates, achieving a 67 % reduction in the degree of infection compared to the infected control. Overall, isolates Ory09, Ory59, Ory29, and Ory22 were found to be more effective in suppressing the physiological negative effects caused by *B. glumae* in both varieties at a 95 % significance level ($p > 0.05$).

Among the observations in each treatment, a reduction in growth, deterioration of the plumula, and few root hairs can be seen in the seedlings challenged by *B. glumae*. On the other hand, in the endophyte pre-treatments, seedlings with longer roots, more root hairs, better coleoptile development, and emergence of the first leaves were observed. These observations demonstrate the potential of endophytic

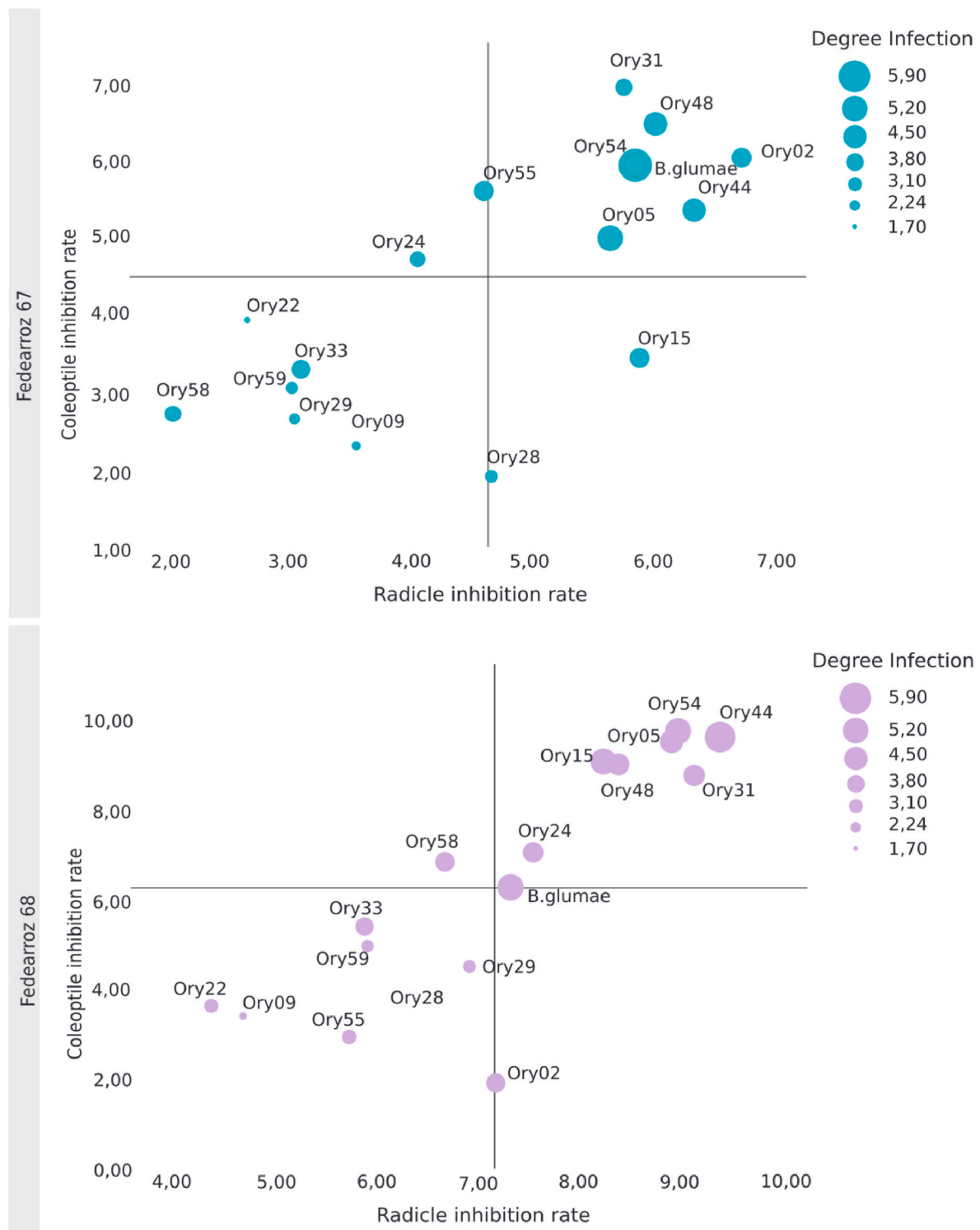


Fig. 3. Evaluation of the impact of endophytic bacteria on the reduction of Coleoptile inhibition rate (CIR) (axis Y), Radicle inhibition rate (RIR) (axis X), and Degree of infection (DI) (circle size) in seeds infected with *Burkholderia glumae*. The scatter plot illustrates the correlation between the three variables for both F67 (top) and F68 (below) rice varieties.

bacteria to mitigate the negative effects of *B. glumae* on rice seedlings, highlighting their biocontrol efficacy (Fig. 4).

3.5. Phylogenetic analysis of endophytic bacteria

The phylogenetic results show the separation of four major taxonomic groups, with the bacterial morphotypes placed mainly in two

groups (Fig. 5). The first group identifies the morphotypes Ory15, Ory22, Ory33, Ory29, Ory28, Ory02 as *Bacillus cereus*, and Ory59 as *Bacillus pumilus*. The second group corresponds to the bacterial class γ -proteobacteria, with the morphotype Ory22 homologous to the genus *Acinetobacter* sp.; Ory58 homologous to *Pantoea* sp. and the morphotypes Ory39, Ory20 and Ory55 belonging to the genus *Enterobacter* sp. Results of the phylogenetic analysis using primers F948 β and R1492,

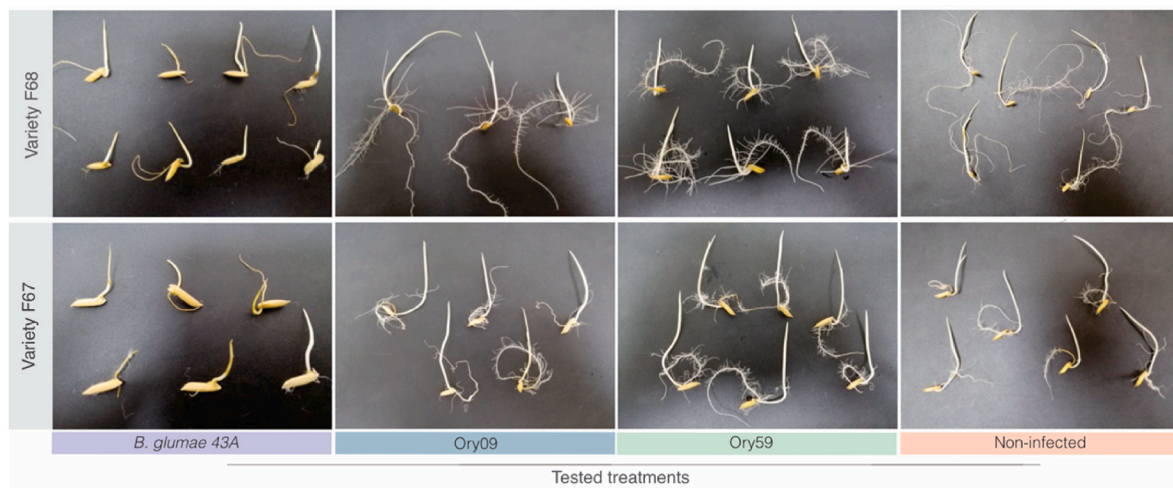


Fig. 4. Biocontrol activity of endophytic bacteria against both physiological and symptomatic adverse effects by *Burkholderia glumae* on rice seeds of Fedearroz 68 (top row) and Fedearroz 67 (bottom row) and the effects of endophytes Ory09 and Ory59 to combat the disease.

specific for the beta-proteobacterial class, indicate a homology with a branch support of 91 % identity with the morphotypes Ory09 as *Herbaspirillum seropedicae*. Alternative sequences belonging to the fimicute, alpha, and beta proteobacterial classes were included as outgroups to confirm the homology and affinity of the primers for the β -proteobacterial class.

4. Discussion

Burkholderia glumae, a recognized phytopathogen, has been identified as a major contributor to substantial losses in rice crops [11]. Research on this pathogen dates back to 1967, with investigations aimed at understanding the virulence factors involved in the infection process and the molecular patterns of evasion of the plant defense response that *B. glumae* uses to cause bacterial leaf blight in rice. These studies have confirmed that *B. glumae* can infect various plant tissues, ultimately leading to bacterial panicle blight disease in rice crops [49,50]. Recent research endeavors have been dedicated to exploring this pathosystem further, with a focus on developing more effective strategies for disease control than those currently in use [51,52]. Despite these efforts, our understanding of the disease impact on rice seeds remains limited. Consequently, it is imperative to comprehensively assess the pathogenic effects of *B. glumae* within the seeds to develop effective prevention and management strategies for this disease in the field [4].

The investigation has revealed that the *B. glumae* strain has a profound impact on the physiological processes during seed germination. Iiyama et al. [53] evaluated the virulence levels exhibited by various pathogenic strains on seedlings; this analysis revealed a direct correlation between the concentration of phytotoxins produced by *B. glumae* and the inhibited elongation of both shoots and roots. As a result, Iiyama et al. [53] concluded that phytotoxins could be the primary virulence factor responsible for the physiological damage observed during seed germination. Notably, mutation studies have identified two key proteins, TRP-1 and TRP-2, involved in the production of toxoflavin, which is considered the primary virulence factor of *B. glumae* [54].

The results of this study show that not all *B. glumae* strains tested induced symptoms by affecting plant physiology. For example, strain 4026-1 was the least aggressive in both cultivars, especially in F68. Similar results were obtained by Iiyama and co-workers [53], who evaluated the level of virulence of several pathogenic strains on seedlings and panicles, considering the concentration of phytotoxins expressed by each strain. Their results show that strains with higher production of phytotoxins expressed symptoms related to reduced shoot and radicle elongation, and those with lower production of toxoflavin

reduced their symptoms and tissue damage. However, studies have shown that mutations in toxoflavin do not necessarily limit the virulence of a strain but rather depend on other factors inherent to virulence [26]. Moreover, disease severity is influenced by the genetic characteristics of the crop varieties [49,55,56]. In the results obtained, strain 43A caused greater severity in terms of tissue damage in rice seedlings of cultivar F68. Research on the pathogenicity of *B. glumae* and its impact on seedlings has indicated that infection by the pathogen in susceptible genotypes is preceded by extensive colonization of vascular tissue, leading to decreased shoot development, chlorosis, decay, and destruction of vascular tissue [57]. Studies have demonstrated that the expression of quorum-sensing molecules activates high concentrations of the pathogen in vascular tissue. In *B. glumae*, acyl homoserine lactone (AHL) is the main cell-to-cell signaling mechanism and regulates the expression of genes responsible for toxoflavin. The study examines the impact of quorum sensing on various virulence factors, including the production of lipases that cause surface damage to plant tissue and polygalacturonase, which breaks down tissue. Both enzymes are responsible for soft tissue rot in other plant species [11,58]. Méndez [59] conducted a series of studies using various rice varieties to investigate the impact of *B. glumae* inoculum concentration. These studies unveiled that concentrations exceeding 10^6 colony-forming units per milliliter (cfu/mL) intensified the severity of the disease in the F67 variety. Similarly, the findings described by Flórez and Vélez [38] indicated that even tolerant rice varieties could become susceptible to high concentrations of *B. glumae*. This suggests that the severity of the disease is likely influenced by the initial concentration of the pathogen. Furthermore, these results underscore the necessity of thoroughly examining each factor involved in the plant-pathogen interaction to comprehensively characterize the pathogenicity of panicle blight caused by *B. glumae* across different rice varieties [8,36,37,60].

Biological assays involving the inoculation of seeds with a beneficial agent followed by subsequent infection with a pathogen are commonly employed to evaluate the biocontrol potential of endophytic bacteria. This model of defense induction is known as priming. Priming is a form of defense in which the plant perceives external stimuli that prepare it to mount a stronger defense response against future attacks [61,62]. These stimuli can come from a variety of sources, including the use of beneficial microorganisms. It has been shown that interaction with beneficial microorganisms activates JA and ET-dependent signaling pathways that lead to the induction of systemic resistance in the plant under pathogenic attack [63]. In the context of this study, it was found that 16 endophytic bacteria exhibited growth-promoting traits, including nitrogen fixation activity and phosphate solubilization, among others.

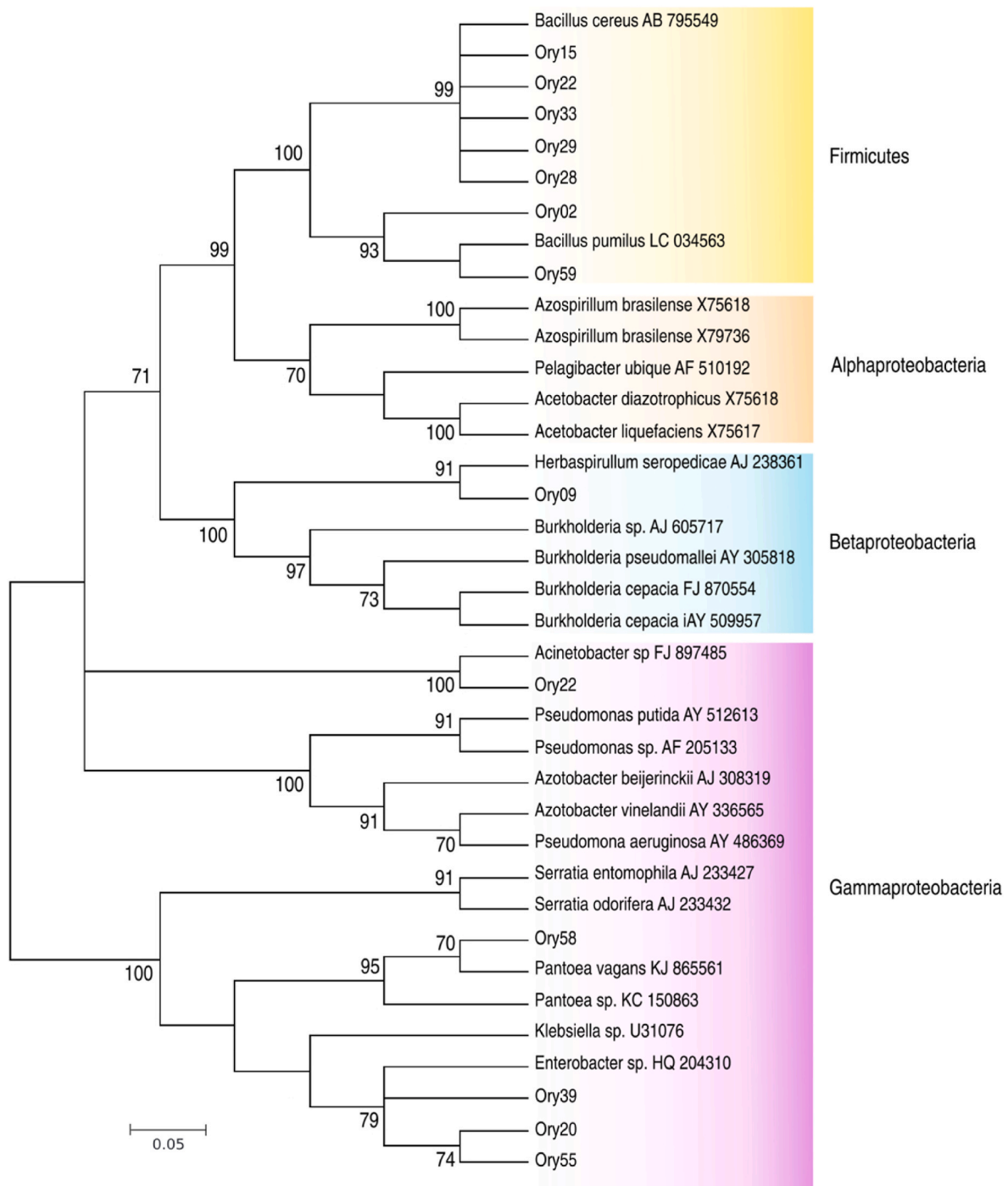


Fig. 5. Phylogenetic tree from 16s rDNA sequencing identifying the species of bacterial endophytes evaluated in this study. The scale bars represent 0.05 substitutions per nucleotide position.

Additionally, the results demonstrated an increase in biomass, as determined by the dry weight of the tissues inoculated with endophytic bacteria. These findings are in alignment with existing studies that have reported a positive correlation between phosphate-solubilizing endophytes and enhanced biomass, with approximately 86 % of the endophyte population capable of phosphate solubilization contributing to this phenomenon [64,65]. From these results and insights drawn from other research, it can be concluded that endophytes employ various mechanisms depending on factors such as species, genotypes or strains and environmental growth conditions, to exert their beneficial effects on the host plant [66–68].

This study successfully identified five endophytic bacteria Ory22, Ory29, Ory09, Ory59, and Ory33 with the potential to mitigate the pathogenic effects of *B. glumae* on seed germination. These identified strains demonstrated a reduction in growth inhibition rates and disease severity. Consequently, endophytic bacteria are proposed as a viable biocontrol alternative for managing *B. glumae*. Numerous studies have provided evidence that endophytic bacteria can effectively control various crop diseases. For instance, Pedraza et al. [26] investigated rhizospheric bacteria with inhibitory activity against *B. glumae* and found that using concentrations of 10^8 colony-forming units per milliliter (cfu/mL) provided protection against pathogen infection

without any discernible difference in seed growth between the treated and healthy control groups. The findings of the present study indicate that endophytic bacteria are capable of mitigating the symptoms caused by *B. glumae*, without any adverse effects on seedling physiology and growth. It should be noted that, in contrast to rhizospheres, endophytic bacteria, due to their close interaction with various metabolic processes in the host plant, are an exceptional option for controlling crop field diseases.

Several studies have reported the ability of bacteria to modulate molecular signaling pathways during the infection process [20,25,31,32]. The phylogenetic results identified species and genera of bacteria reported in the literature as possible inducers of a defense response against pathogen attack. Among these is *Bacillus cereus*, which was identified as a potential growth promoter and has been described as a resistance inducer in recent research in rice for its ability to reduce the incidence of the pathogen *Sarocladium oryzae* by inhibiting mycelial growth, expressing antibiotic-related genes, and increasing the enzymatic activity of defense-related genes in plants [69]. *B. cereus* has been extensively studied for its ability to activate immune responses as measured by PAMS [70] expression of PR genes in *Fragaria × ananassa Duch* [71], and even induction of resistance, not only *in vitro* but its potential to reduce disease incidence has been observed in the greenhouse and the field [72]. *Bacillus pumilus* has been identified within the genus *Bacillus* for its potential growth-promoting activity and ability to confer protection to plants under stressful conditions, including induction of systemic resistance (ISR) in various crops such as maize [73], tomato, watermelon, pepper and others [74]. On the other hand, the results of the molecular identification of the bacteria showed the presence of endophytic bacteria belonging to the genus *Acinetobacter* sp. of the class of Gammaproteobacteria. This genus is potentially important in agriculture and is one of the most important PGPR bacteria [75,76]. One of the bacterial genera identified was *Pantoea*, related to Ory 58. Although research on this genus has mainly focused on its potential as PGPB, some research has demonstrated its biocontrol potential against pathogens by showing the expression of defense genes in an infectious process [77]. The results show that Ory58 was the only endophyte that exhibited PGPR activities *in vitro*, a significant increase in the vigor index of both rice varieties, and a reduction in symptoms in the F67 variety. These findings underscore the significance of *Pantoea* sp. In the bioprospecting of economically important crops. On the other hand, three endophytic isolates, Ory39, Ory20, and Ory55, were found to be related to the genus *Enterobacter* sp. This genus comprises a diverse range of bacterial species with the potential to promote growth. In recent years, the *Enterobacter* genus has been highlighted for its ability to mitigate the effects of abiotic stresses on agriculturally important crops, including salt stress, drought stress, and stress caused by phytotoxic compounds [78]. Finally, *Herbaspirillum seropedicae* (Ory09) was identified as the endophyte isolate selected to evaluate the reduction of the severity index. Bacteria of this genus are of great agricultural interest due to their ability to produce molecules that positively affect physiological processes and confer plant resistance to environmental stress and pathogens [79]. Among these molecules, the most studied in *Herbaspirillum* is LPS. These transmembrane molecules have been shown to facilitate penetration into plant tissues, induction of ROS responses, induction of proteins related to pathogenesis, and induction of defense responses, among others. Studies using molecular mass coupled chromatography techniques have identified the composition of LPS *Herbaspirillum*, including 3-deoxy-2-manno-octulosonic acid, lauric acid, and myristic acid, among others [80]. LPS has been described as a potential inducer of systemic responses that protect against future pathogen attacks, a process of priming [81]. However, the molecular mechanisms involved in defense modulation by *Herbaspirillum* sp. against *B. glumae* in rice are unknown. The results described in this study demonstrate the efficacy of *H. seropedicae* (Ory09) in reducing the severity of bacterial blast disease on seeds and seedlings. Consequently, it is imperative to continue conducting investigations aimed at

elucidating the diverse mechanisms through which endophytic bacteria can counteract the pathogenic effects induced by *B. glumae* in rice crops. This research will facilitate the development of effective alternatives for disease control in the field. The results of the present study open the way to a deeper understanding of the molecular mechanisms by which endophytes mediate a defense response against *B. glumae*. It would also be interesting to evaluate the strains used here not only at the seedling level but also in panicles in the field and to observe whether the ability of endophytes to reduce the severity of *B. glumae* is persistent in the advanced stages of crop development.

CRedit authorship contribution statement

Zafiro Barraza Román: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hector Alejandro Rodríguez Cabal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **María Cecilia Lara Mantilla:** Supervision, Project administration, Formal analysis, Conceptualization. **Alexander Pérez Cordero:** Supervision, Project administration, Funding acquisition, Conceptualization. **Zulma Isabel Monsalve Fonnegra:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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