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RESEARCH

Detection of *Pseudomonas fuscovaginae*, *Burkholderia glumae* and *Pseudomonas syringae* in rice seeds and symptomless plant material by BIO-PCR

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ABSTRACT

Rapid detection and accurate identification of seed-borne bacteria are critical steps to prevent pathogen dissemination. Molecular-based methods such as PCR have greatly improved detection. However, plant inhibitors can interfere its detection. Despite, pre-enriching bacteria on agar media (BIO-PCR) can increase the sensitivity and reduce the inhibitor's effects. Three primer sets were designed for detecting *Burkholderia glumae* (Bg-BIO), *Pseudomonas fuscovaginae* (Pfus-BIO) and *Pseudomonas syringae* (Ps-BIO), and specificity and sensitivity of conventional PCR assessed. Detection limits of PCR and BIO-PCR were compared, and so were pathogen detection in artificially-infected seeds and in symptomless plant material. It was found that PCR using the designed primers were specific (detection limits 4-400 pg/µL of target DNA). Pfus-BIO and Ps-BIO were highly sensitive and allowed increasing detection limits significantly compared with conventional PCR. Conversely, Bg-BIO was only 10-times more sensitive than conventional PCR. Bacteria were efficiently detected by PCR and BIO-PCR from artificially inoculated seeds, the same results achieved for both methods with large pathogen amounts and macerated seed extracts. *P. fuscovaginae* and *B. glumae* were detected in 26 (87%) and 18 (60%) out of the 30 symptomless plant material samples tested, whereas *P. syringae* was undetected in any of the samples. Conventional PCR did not allowed target amplification of any sample. In summary, the designed BIO-PCR tests are reliable and efficient tools for diagnosing *B. glumae*, *P. fuscovaginae*, and *P. syringae*, enabling to sow only bacteria-free propagating material and moving it from one place to another.

Keywords: bacterial pathogen, *Oryza sativa* L., molecular detection, seed-borne bacteria, PCR

RESUMEN

Detección de *Pseudomonas fuscovaginae*, *Burkholderia glumae* y *Pseudomonas syringae* en semillas de arroz y material vegetal asintomático mediante BIO-PCR. La detección rápida y la identificación precisa de las enfermedades bacterianas transmitidas por semillas, mediante técnicas moleculares como el PCR, son pasos críticos para su manejo. Sin embargo, ciertos inhibidores derivados de las plantas interfieren en su detección. Tales inconvenientes se pueden eliminar mediante el enriquecimiento previo en medios de cultivo (BIO-PCR), e incrementar la sensibilidad de los ensayos y reducir los efectos de los inhibidores. En este trabajo se diseñó tres parejas de cebadores para la detección de *Burkholderia glumae* (Bg-BIO), *Pseudomonas fuscovaginae* (Pfus-BIO) y *Pseudomonas syringae* (Ps-BIO), y se evaluó la especificidad y la sensibilidad de los PCR convencionales. Se comparó los límites de detección de los PCR y los BIO-PCR, y la detección de patógenos en semillas infectadas artificialmente y en material vegetal asintomático. Se generó PCR específicos (límites de detección de 4-400 pg/µL) de ADN diana. Pfus-BIO y Ps-BIO incrementaron considerablemente los límites de detección en comparación con los PCR convencionales, mientras que con Bg-BIO fue solo de 10 veces. Se detectó a las bacterias eficientemente por PCR y BIO-PCR a partir de semillas inoculadas artificialmente, con niveles de detección similares por ambos métodos, a concentraciones elevadas de los patógenos y mediante maceración de las semillas. En 30 muestras, se detectó *P. fuscovaginae* en 26 (87 %) y *B. glumae* en 18 (60 %), y *P. syringae* no fue detectada. Los BIO-PCR diseñados son herramientas confiables y eficientes para el diagnóstico de *B. glumae*, *P. fuscovaginae* y *P. syringae*, y permiten implementar medidas de manejo y contención de estos patógenos.

Palabras clave: patógeno bacteriano, *Oryza sativa* L., detección molecular, bacterias transmitidas por semillas, PCR

How to cite (Vancouver style):
Plasencia-Márquez O, Rivero D, Martínez-Zubiaur Y. Detection of *Pseudomonas fuscovaginae*, *Burkholderia glumae* and *Pseudomonas syringae* in rice seeds and symptomless plant material by BIO-PCR. Biotecnol Apl. 2023;40(1):1201-7.

Introduction

Global economic disruptions caused by COVID-19 pandemic resulted in an estimated 97 million more people living in poverty in 2021 compared to 2019, leaving tens of millions more people hungry and malnourished [1]. In this context, rice (*Oryza sativa* L.) contributes to improving food security by not only fighting hunger but also acute malnutrition; and continues to be a major food staple for more than 3.5 billion people. According to FAO reports, world rice utilization in 2021/22 was pegged at 520.8 million tonnes, up 1.5 percent from the previous season, with a global rice intake at close to 54 kg per person [1]. Thus, global sanitary emergence, population growth and nutritional demands require a higher increase in rice production. That is independent of challenges such as climate change, environmental

1. FAO. Crop Prospects and Food Situation-Quarterly Global Report No. 2. July, 2021. Rome: FAO. 2021 [cited 2022 Sep 18]. Available from: https://doi.org/10.4060/cb5603en

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stress, and diseases; and it is more relevant in regions with limited resources to mitigate the impacts of such events [2].

In fact, Cuba is one of the main consumers of rice in Latin America, with an annual per capita consumption over 70 kg [3]. Food security and nutrition are high priorities for the Cuban Government, as outlined in its national plan for economic and social development through 2030 [4], although the impact of the Coronavirus Disease 2019 (COVID-19) pandemic. Despite the strategies to promote rice production, only 50% of the demand is covered because the average agricultural yield remains close to 3 t/ha [5], even with high productive potential cultivars. Several factors are influencing this yield, among them, pests incidence among the most significant ones [6]. For instance, *B. glumae* was detected in imported rice seeds and also in productive areas in Cuba [7], and strict quarantine measures for control and eradication had to be reinforced, these pathogens included in the official List of Quarantine Pests of the Republic of Cuba [8]. Thus, testing procedures in planting materials are required to prevent their introduction or to restrict their spread in a geographical location.

In this setting, the contention of most common rice-infecting bacterial pathogens, *Pseudomonas fuscovaginae* and *Burkholderia glumae* remains critical. These are the causal agents of brown sheath rot and bacterial panicle blight, respectively [9, 10]. Together with piriculariosis and sheath blight, they account for huge economical losses in rice crops worldwide [11]. Other diseases associated with *Pseudomonas syringae* complex have emerged in recent decades; such are the cases of bacterial sheath rot of rice, caused by *P. syringae* pv. *syringae* van Hall and bacterial halo blight, caused by *Pseudomonas syringae* pv. *oryzae* (ex Kuwata) Young [12, 13].

These bacterial diseases can be transmitted by contaminated rice seeds and other plant materials. Seeds are passive carriers and may even be responsible for the introduction of exotic diseases to new areas [14]. That's why its rapid detection, accurate identification and seed treatment are critical steps in formulating sustainable management of plant bacterial diseases. Therefore, the use of pathogen-free seeds is recommended and, consequently, seeds' lots are routinely tested by the regulatory agencies and seed companies [14]. Furthermore, accurate detection of pathogens in symptomless plant material is essential, particularly at early infection stages, to prevent pathogen spread and disease proliferation.

Molecular testing techniques have provided adequate tools for the simultaneous detection of plant pathogens and its identification, including PCR in different formats (real-time PCR, multiplex PCR, nested-PCR and BIO-PCR) [15]. These techniques are time-saving, highly specific, and do not require pathogen isolation, supporting fast and precise diagnoses to provide high quality seeds. However, plant inhibitors interfere with most of these tests, while the population of these target pathogen in seeds and symptomless plant material is often lower than for other bacteria [16]. Advantageously, BIO-PCR includes a first step of biological amplification on liquid or solid media, increasing, which increases the target DNA amount and remove the inhibitors' interference, also providing a viable culture of the target organism [16]. Therefore, this work was aimed to design and evaluate three BIO-PCR systems to detect *B. glumae, P. fuscovaginae*, and *P. syringae* in rice seeds and symptomless plant material.

Materials and methods

Bacterial strains and growing conditions

Pseudomonas and non-*Pseudomonas* strains used in this study (Table 1) were obtained from the collections of the Plant Bacteriology Laboratory, National Center for Animal and Plant Health (CENSA), Cuba, and the Bacteriology Laboratory of the International Center of Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy. The strains were maintained on King's medium B (KB) and Luria-Bertani Agar medium (LBA), respectively, at 4 °C for routine use, and in 18 % glycerol at –80 °C for long term storage. Three plant pathogenic strains were used as templates to evaluate the PCR and BIO-PCR protocols: *Pseudomonas fuscovaginae* UPB0736 [17], *Burkholderia glumae* ATCC33617^T [18], and *Pseudomonas syringae* pv. *syringae* B728a [19]. *Pseudomonas* strains were grown in KB medium at 28 °C for 24 h and *B. glumae* was grown in LBA at 28 °C for 48 h.

Primer design

Primer sets were designed using partial sequences of genes *pfsI* of *P. fuscovaginae*, *recA* of *P. syringae* pathovars and *rpoD* of *B. glumae*. Sequences available in the GenBank (National Center for Biotechnology Information, NCBI, https://www.ncbi.nlm.nih.gov) were aligned using MEGA v7 software [20]. The consensus sequences were used to design primers with Primer3 Plus program (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) [21]. To assess primer specificity, in silico analyses were developed using NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with default options.

Table 1. Bacterial strains used for BIO-PCR assay development

Bacterial strain	Origin	Host plant	Source
Pseudomonas fluorescens A9, A36, A63, PR1	Cuba	*Oryza sativa* L.	PBLC
Pseudomonas putida A90, A91, A10, A11, A12	Cuba	*Oryza sativa* L.	PBLC
Pseudomonas fuscovaginae UPB0736	Madagascar	*Oryza sativa* L.	BLI
Pseudomonas syringae pv. syringae B728a	Wisconsin, USA	*Phaseolus vulgaris* L.	BLI
Burkholderia glumae ATCC33617^T	Japan	*Oryza sativa* L.	BLI
Serratia fonticola E2309	Venezuela	*Oryza sativa* L.	BLI
Bacillus amyloliquefaciens E1101	Venezuela	*Oryza sativa* L.	BLI
Aeromonas diversa E2102	Venezuela	*Oryza sativa* L.	BLI
Pseudomonas mendocina E1108	Venezuela	*Oryza sativa* L.	BLI
Delftia tsuruhatensis E2330	Venezuela	*Oryza sativa* L.	BLI
Pseudomonas pseudoalcaligenes E12025	Venezuela	*Oryza sativa* L.	BLI
Pseudomonas aeruginosa S2.8	Cuba	*Glycine max* L.	PBLC
Bacillus pumilus E2315	Venezuela	*Oryza sativa* L.	BLI
Acidovorax sp. AG323	Italy	*Oryza sativa* L.	BLI
Burkholderia cepacia AG1004	Italy	*Oryza sativa* L.	BLI
Burkholderia sp. AG845	Italy	*Oryza sativa* L.	BLI
Comamonas sp. AG1104	Italy	*Oryza sativa* L.	BLI
Methylobacterium AG1084	Italy	*Oryza sativa* L.	BLI
Ralstonia sp. AG522	Italy	*Oryza sativa* L.	BLI

PBLC: Laboratory of Plant Bacteriology, CENSA, Cuba. BLI: Bacteriology Laboratory, International Center for Genetic Engineering and Biotechnology, Trieste, Italy.

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DNA extraction

Genomic DNAs were obtained from overnight (ON) cultures, following Doyle and Doyle protocol [22], and quantified using a ThermoScientific Nanodrop 1000 Spectrophotometer. When DNA from a defined number of cells was required, one millilitre from an overnight culture, adjusted to 1.0 OD_{600nm} (10^8 c.f.u./mL), was processed by heat shock, according to the protocol of Moore *et al*. [23].

Conventional PCR assessment

In vitro specificity and sensitivity of conventional PCR were evaluated. Genomic DNA, extracted from *Pseudomonas* and non-*Pseudomonas* strains listed in Table 1, were used to test primer specificity. Sensitivity was tested using serial dilutions of genomic DNA (40 ng/µL-4 fg/µL) and dilutions of DNA extracted from a defined number of cells (10^8 c.f.u./mL). The PCR assays were performed in a MJ Mini Personal Thermalcycler (BIO-RAD). The final volume of 25 µL contained: 1× Green GoTaq® Reaction Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM primers, 1.25 u GoTaq® G2 Flexi DNA Polymerase (Promega), and 1µL of DNA. The program included an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C (*Pseudomonas* sp.) or 56 °C (*B. glumae*) for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 10 min. Ten microliters of the PCR product were separated by electrophoresis on 1 % agarose gel in 1× TAE buffer (40 mM Tris-base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) at 80 V. The gel was stained with Invitrogen™ SYBR™ Safe DNA Gel Stain.

BIO-PCR assays

The seeds (cultivar Baldo) were disinfected with tap water for 30 min, sodium hypochlorite (2-3% v/v) for 30 min, and serial washings were applied with sterile distilled water. Seed-water extracts (SWE) were prepared by soaking 22.5 g of disinfected rice seeds overnight at 4 °C in 50 mL of NaCl 0.85 % saline solution plus 0.01 % Tween-20. Aliquots of SWE (0.1 mL) were plated in triplicates onto LBA plates to confirm the absence of target bacteria. Stocks of seed extracts were also prepared, containing expected concentrations of pathogens (0.1-10^8 c.f.u/mL). These stocks were made starting from overnight bacterial cultures (OD_{600nm} = 1, equivalent to 10^8 c.f.u./mL), serially diluted in the SWE. Besides, a second stock was made by diluting the bacterial cultures in NaCl 0.85 % saline solution, to compare PCR detection limits. Each stock was plated (0.1 mL) in triplicates onto LBA plates, and incubated at 30 °C for 48 h. Thereafter, plates were washed with 3 mL of sterile distilled H_2O, and samples were processed by heat shock [23]. Two microliters each were used to perform the PCR assays described above, using specific primers. The assays were repeated twice.

Pathogen detection in artificially infected seeds

Pathogen detection in artificially infected seeds by conventional PCR and BIO-PCR was evaluated. Seeds were disinfected as described above and were incubated by shaking at 100 rpm in bacterial culture suspensions (10^8 c.f.u./mL-large pathogen amount or 10^2 c.f.u/mL-small pathogen amount). Seeds were harvested by removing the bacterial suspensions and dried at 30 °C. Afterward, they were mixed with healthy seeds in different proportions (1:20-10:20, equivalent to 1-10 infected/g of seeds). Surface-sterilized seeds were used as negative controls.

The 20 seeds (~1 g) of each proportion and negative controls were soaked overnight in 50 mL of NaCl 0.85 % saline solution plus 0.01% Tween-20 at 4 °C. Then, 2 mL of the resultant extracts (non-macerated seeds) were kept for later. The seeds were macerated with mortar and pestle and incubated at 4 °C for other 10 min. A volume of 100 µL of the resultant extracts, from the macerated and non-macerated seeds, were plated onto LBA in triplicates, and incubated at 30 °C for 48-72 h. Washes were performed with 3 mL of sterile distilled H_2O, were processed by heat shock and 2 µL were used for BIO-PCR, as described above. Also, 1 mL of the resultant extracts (macerated and non-macerated) was used for DNA extraction [22] and conventional PCR.

Pathogen detection in symptomless leaves from inoculated rice seeds

Rice seeds were artificially inoculated with bacterial suspensions (10^8 c.f.u./mL) as described above. They were kept at 30 °C and high humidity for one week to promote germination and they were then seeded in Falcon tubes containing Hoagland's solution [24] supplemented with 0.25 % agar. The tubes were incubated at 30 ± 6 °C and high relative humidity (close to 100 %) under photoperiods of 16 h light/8 h darkness by using 400 V lamps. In order to evaluate pathogen detection, 30 symptomless samples (1 g) for each pathogen were taken after five days of sowing.

Plants resulting from healthy seeds were used as negative controls. Samples (including roots and leaves) were serially washed with sterile distilled H_2O, macerated on a mortar with a pestle, and incubated overnight in 3 mL of NaCl 0.85 % saline solution plus 0.01 % Tween-20 at 4 °C. Then, 100 µL were used for BIO-PCR, developed as described above. One milliliter was used for DNA extraction [22] and conventional PCR. The quality of the DNA used for conventional PCR was checked by amplification of 16S rDNA using the universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rp2 (5'-ACGGCTACCTTGTTACGACTT-3') [25].

Results

Conventional PCR assessment

Three primer sets Bg2-Fw/Bg2-Rev, Pfusco-Fw/Pfusco-Rev, and Pss-Fw/Pss-Rev were designed to detect *B. glumae*, *P. fuscovaginae*, and *P. syringae*, respectively. Oligonucleotide sequences, putative product sizes and their corresponding target genes are listed in Table 2.

These primers showed high *in silico* specificities and aligned only with sequences corresponding to target species. Also, *in vitro* specificities were demonstrated by conventional PCR. In all the cases, the expected PCR amplicons were obtained for with positive controls (*B. glumae* ATCC33617^T, *P. fuscovaginae* UPB0736, and *P. syringae* pv. *syringae* B728a).

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None of the other non-target strains produced a detectable PCR product.

The analytical sensitivity of each PCR, determined by testing serial dilutions of target DNAs, containing 40 ng/µL-4 fg/µL showed the same thresholds for *B. glumae* and *P. fuscovaginae* PCR (400 pg/µL). Noteworthy, the *P. syringae* PCR was more sensitive, being able to detect up to 4 pg/µL of its target DNA (Figure 1).

BIO-PCR assessment

BIO-PCR protocols using the designed primers were developed to detect the pathogens in rice seeds. Detection limits of conventional PCR (Pfus-C, Ps-C and Bg-C) and BIO-PCR (Pfus-BIO, Ps-BIO and Bg-BIO) were compared using known numbers of pathogen cells. Pfus-BIO and Ps-BIO, in respect to conventional PCR, were highly sensitive and allowed increasing detection limits significantly (10^4-fold) when pathogen dilutions were made in SWE (Table 3). On the other hand, Bg-BIO was only 10-fold more sensitive than Bg-C.

It was confirmed the absence of target bacteria in SWE when dilutions of the pathogen were made. When bacterial cultures (10^8 c.f.u./mL) were diluted in NaCl 0.85 % saline solution, BIO-PCR thresholds were 1 c.f.u./mL for all pathogens. Thresholds in SWE and NaCl 0.85 % saline solution were the same (1 c.f.u./mL) only for *P. syringae*. The detection limit was slightly lower in SWE (10-fold) for *P. fuscovaginae* and noticeably lower for *B. glumae* (10^4-fold).

Pathogen detection in artificially infected seeds

Pathogen detection from artificially inoculated rice seeds was evaluated by BIO-PCR and conventional PCR. When a large pathogen amount (10^8 c.f.u./mL) was used to infect the seeds (Figure 2), BIO-PCR from extracts of macerated and not-macerated seeds detected the pathogen even when only one seed/g was infected (1:20), using large amounts (10^8 c.f.u./mL) of pathogens to infect seeds. On the other hand, pathogens were detected up to 1:20 by conventional PCR only in extracts of macerated seeds, and in two or more seeds infected (two infected seeds/g) starting from non-macerated material. Noteworthy, pathogens were equally detected using conventional PCR or BIO-PCR from large pathogen amounts and macerated extracts.

At smaller pathogen amounts (10^2 c.f.u./mL), BIO-PCR from extracts of macerated and non-macerated seeds, and PCR from macerated material, supported detection from at least four infected seeds/g with *P. fuscovaginae* or *P. syringae*, and six infected seeds/g with *B. glumae*. The three pathogens were detected in conventional PCR assays in non-macerated seeds from at least six infected seeds/g (data not shown).

Pathogen detection in symptomless leaves from inoculated rice seeds

The detection capacity of BIO-PCR was evaluated using infected but symptomless plant material as a starting point, and further compared with conventional PCR. After five days of planting, target DNAs were amplified in 18/30 (60 %) samples and 26 out of 30 (87 %) of symptomless leaves coming from seeds

Table 2. List of primers used in PCR and BIO-PCR assays

Primer	Sequence (5´-3´)	Tm (° C)	Product size (bp)	Target gene	Target organism
Bg2-Fw Bg2-Rev	TTCGTGTGGACGAACTCGTT CCGGACCTCATCCACCTG	65 64	395	RNA Polymerase sigma factor (*rpoD*)	*Burkholderia glumae*
Pfusco-Fw Pfusco-Rev	AGCAGACAAGGCTTTTATCGT CGCTACAAGGTCTTCGTCGA	63 65	510	N-acyl-L-homoserine lactone synthetase (*pfsI*)	*Pseudomonas fuscovaginae*
Pss-Fw Pss-Rev	GGGTGCTACCTGTGCCTT CCAGGAACTTGGCCGAGTT	65 65	671	Recombinase A protein (*recA*)	*Pseudomonas syringae*

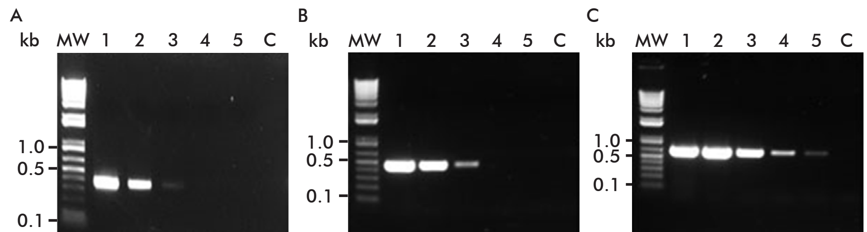

Figure 1. Analytic sensitivity of conventional PCR for the identification of *Burkholderia glumae*, *Pseudomonas fuscovaginae* and *Pseudomonas syringae* in rice seeds. DNA agarose gel electrophoresis (1 %) run at 120 V. A) *B. glumae*. B) *P. fuscovaginae* C) *P. syringae*. MW: 1 kb Plus DNA Ladder (Thermo Fisher Scientific). Lanes 1-5: tenfold dilutions of target DNA (40 ng/µL-4 pg/µL). C: negative control (water).

Table 3. Analytic sensitivity of conventional PCR and BIO-PCR for pathogenic bacteria detection from rice seeds

PCR assay	Number of cells (c.f.u./mL)								
	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10	1
Bg-C	+	+	+	+	–	–	–	–	–
Bg-BIO	+	+	+	+	+	–	–	–	–
Bg-BIO (SS)	+	+	+	+	+	+	+	+	+
Pfus-C	+	+	+	+	–	–	–	–	–
Pfus-BIO	+	+	+	+	+	+	+	+	–
Pfus-BIO (SS)	+	+	+	+	+	+	+	+	+
Ps-C	+	+	+	+	+	–	–	–	–
Ps-BIO	+	+	+	+	+	+	+	+	+
Ps-BIO (SS)	+	+	+	+	+	+	+	+	+

Bg: *Burkholderia glumae*. Pfus: *Pseudomonas fuscovaginae*. Ps: *Pseudomonas syringae*. C: conventional PCR. BIO: BIO-PCR from rice seed water extracts. BIO (SS): BIO-PCR in NaCl 0.85 % saline solution as control.

inoculated with *B. glumae* (Figure 3A-C) and *P. fuscovaginae* (Figure 3D-F), respectively. Otherwise, *P. syringae* remained undetected in any sample of symptomless plant material by BIO-PCR. Furthermore, conventional PCR did not amplified the targets in not any sample, even when DNA quality was checked using universal primers (Figure 3; Table 4).

Discussion

Seeds are the most important source of primary inoculum for many bacterial disease outbreaks, and the availability of quality seeds is often critical to improve food security and reduce poverty in developing countries [26]. Furthermore, a close association of seed-borne pathogens with seeds facilitates their long-term survival, their introduction into new areas and widespread dissemination. The consequences associated with the introduction of exotic pathogens into agricultural ecosystems can be devastating for the industry. Hence, rapid detection and accurate identification of the affecting pathogens before sowing are critical steps

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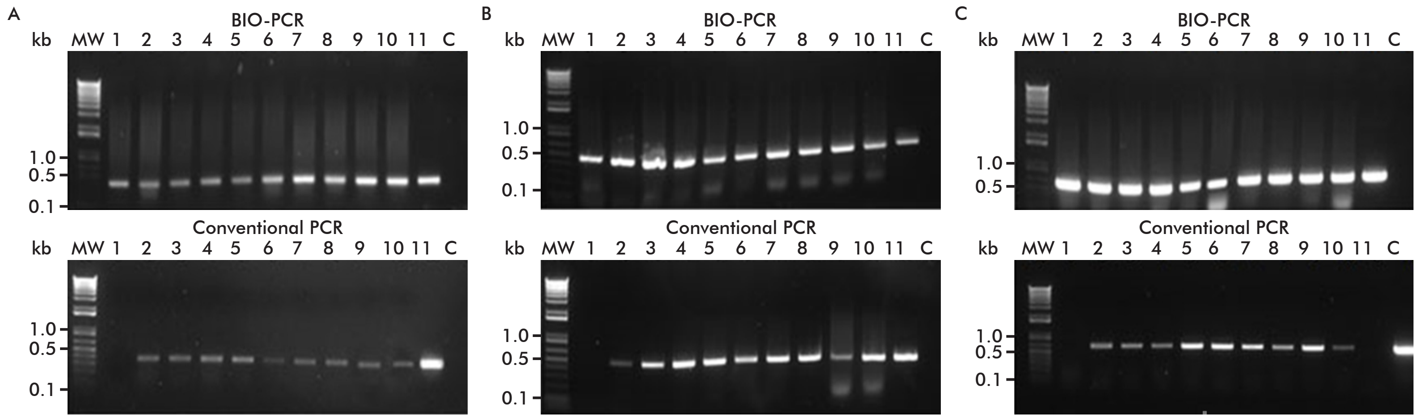

Figure 2. Large pathogen amount detection from artificially-infected rice seeds by PCR assays and agarose gel electrophoresis (1.0 %). A) *Burkholderia glumae*. B) *Pseudomonas fuscovaginae*. C) *Pseudomonas syringae*. MW: 1 kb Plus DNA Ladder (Thermo Fisher Scientific). Lanes 1-5: Pathogen detection from non-macerated infected seeds (1:20-5:20). Lanes 6-10: Pathogen detection from macerated infected seeds (1:20-5:20). Lane 11: Positive control (target DNA). C: Negative control (surface-sterilized macerated seeds).

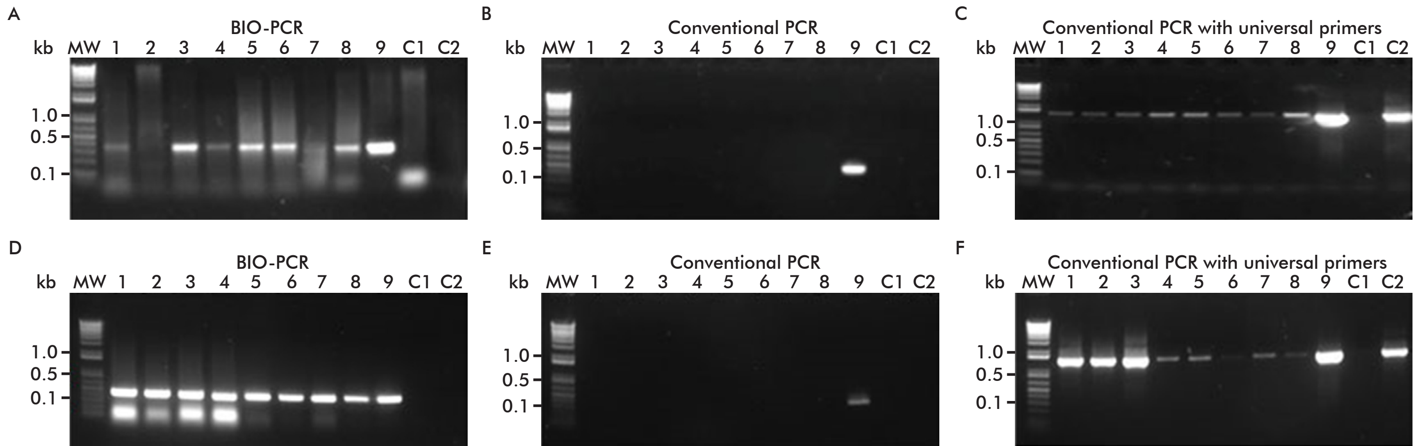

Figure 3. Detection of pathogenic bacteria in some samples of infected but symptomless rice plant material by BIO-PCR or conventional PCR, followed by DNA gel electrophoresis in 1 % agarose gels. A-C) Detection of *Burkholderia glumae* by BIO-PCR, conventional PCR using designed primers, and conventional PCR controls using universal primers, respectively. D-F) Detection of *Pseudomonas fuscovaginae* by BIO-PCR, conventional PCR using designed primers, and conventional PCR controls using universal primers, respectively. MW: 1 kb Plus DNA Ladder (Thermo Fisher Scientific). Lanes: 1-8, asymptomatic plant material from infected seeds; 9, positive control DNA; C1: negative control (water); C2: negative control (plant material isolated from healthy seeds).

to prevent pathogen spreading. And this is the case of the seed-borne pathogens *P. fuscovaginae*, *B. glumae* and *P. syringae*, which cause economically significant diseases in rice worldwide, being included in the official List of Quarantine Pests of Cuba [8].

In this setting, the molecular diagnosis techniques, such as PCR, are valuable tools, being time-consuming, highly specific, and, advantageously, pathogen isolation is not required [27]. Successful detection based on PCR techniques depends upon the specificity of primers and the sensitivity of the assay, as key factors for the official acceptance of newly developed tests for laboratory diagnosis [28].

Considering these, partial sequences of the genes *pfsI* of *P. fuscovaginae*, *recA* of *P. syringae pathovars*, and *rpoD* of *B. glumae*, available in the GenBank databases, were used to design three primer sets. Specificity and sensitivity tests were performed to test whether these primers could be used to detect the target pathogens. The three pathogens were successfully detected with these tests, the assay being specific and sensitive at 4 pg/µL for *P. syringae* and 400 pg/µL for both *P. fuscovaginae* and *B. glumae*.

This remarks the relevance of the availability of pathogen-specific primers for developing PCR-based diagnostic and detection tools. It has been followed by the progressive inclusion of bacterial genomic

Table 4. Sensitivity, amount of pathogenic bacteria and asymptomatic material detected by conventional PCR and BIO-PCR assays

PCR assay	Sensitivity	Total inoculated seeds				Asymptomatic plant material (%)
		Large pathogen amount		Small pathogen amount		
		macerated	non-macerated	macerated	non-macerated	
Bg-C	10^5	1:20	2:20	6:20	6:20	0
Bg-BIO	10^4	1:20	1:20	6:20	6:20	60
Pfus-C	10^5	1:20	2:20	4:20	6:20	0
Pfus-BIO	10	1:20	1:20	4:20	4:20	87
Ps-C	10^4	1:20	2:20	4:20	6:20	0
Ps-BIO	1	1:20	1:20	4:20	4:20	0

Bg: *Burkholderia glumae*. Pfus: *Pseudomonas fuscovaginae*. Ps: *Pseudomonas syringae*. C: conventional PCR. BIO: BIO-PCR.

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DNA sequences at the GenBank database, supporting the development of specific primers using comparative genome analysis [29-31]. Either the 16S rRNA gene or the spacer region between the 16S and 23S rRNA genes of bacterial pathogens have become primary targets for PCR amplification [32].

Otherwise, considering the low rate of molecular evolution of 16S rRNA, alternative gene targets have been tested for primer design, to improve the molecular separation of certain species [33]. Among them, the *rpoD* gene, encoding for the sigma 70 subunit of RNA polymerase, has been estimated to evolve much faster than the 16S rRNA gene, and, therefore, applied for the identification and taxonomic studies of *Pseudomonas* genus [34]. Moreover, the phylogenetic analysis of *recA* (encoding Recombinase A) from a range of bacteria has demonstrated that this gene may be very useful for the differentiation of closely-related species and it has been used for the identification of the *Burkholderia genus* [35, 36]. Here, highly specific PCR-based detection assays were developed with *recA* and *rpoD* as targeting primers for *P. syringae* and *B. glumae*, respectively.

On the other hand, the *pfsI* gene was used for primer design in the *P. fuscovaginae* PCR assay. This gene encodes the N-acyl-L-homoserine lactone synthase from one of the N-acyl homoserine lactone (AHL) quorum sensing (QS) systems of *P. fuscovaginae*, PfsI/R. Strains isolated around the world contained this well conserved system, essential for plant pathogenicity [37]. Then, as shown inhere, *pfsI* can be effectively used in molecular-based *P. fuscovaginae* detection protocols. Additionally, QS genes, such as *lasI* of *Pseudomonas aeruginosa* and *sdiA* of *Salmonella* spp. have been used for specific primer design and pathogen PCR-detection [38, 39].

Despite PCR display advantages and supports the diagnosis of plant pathogenic bacteria, its sensitivity may be affected when samples such as seeds and symptomless plant material are analyzed, due to the smaller pathogen amounts with respect to other bacteria. Besides, PCR cannot differentiate dead from live cells, and it can be affected by inhibitors from the tested samples [40-42]. An alternative method is BIO-PCR, which should eliminate the problem of false negative results, due to PCR inhibitors in the plant or seed extracts, further avoiding false positive results due to dead cells. In fact, the BIO-PCR pre-enrichment phase improves the efficacy and sensitivity of the assay, by increasing the target pathogen population to be tested [42].

The three BIO-PCR protocols designed efficiently detected the target pathogens. When cell dilutions were made in SWE, Ps-BIO and Pfus-BIO assays showed thresholds of 1 and 10 c.f.u./mL, respectively, which were 10^4-fold more sensitive than for the conventional PCR. These results are in agreement with reported standard BIO-PCR sensitivity values, of 5 and 10 c.f.u./mL, about 10 to 100-fold more sensitive than classical PCR [3, 42, 43]. In fact, our assays were even more sensitive. On the other hand, Bg-BIO detected 10^4 c.f.u./mL. This sensitivity was lower than expected for the BIO-PCR method, but it was still 10-times more sensitive than Bg-C. Therefore, more sensitive methods such as real-time PCR or nested-PCR must still be considered, to prevent false negative results when detecting *B. glumae* [41, 44, 45].

Regarding interfering plant inhibitors, these can be eliminated during BIO-PCR and the target bacteria enriched, including other seed-borne saprophytic bacteria, unless using selective media. Furthermore, ubiquitous saprophytic bacteria, often dominant in rice seed samples, overgrow the target bacterium and can inhibit the PCR reaction [46]. Probably, such an effect was observed in the Bg-BIO assay due to the detection limit was remarkably lower (10^4-fold) when pathogen's cells were diluted in SWE instead of NaCl 0.85 % saline solution (absence of saprophytic bacteria). Since *Burkholderia* usually grows more slowly than other organisms frequently found in the samples, it could be overgrown by other seed-borne saprophytic bacteria and can be missed on the medium more easily than *Pseudomonas* strains [47]. BIO-PCR using a semiselective liquid medium together with nested-PCR overcomes this problem and could provide a highly sensitive assay [46]. The lower (10-fold lower in SWE) or no effect observed in Pfusco-BIO and Pss-BIO, respectively, may be due to the fast growth rate of these bacteria, but also to the production of secondary metabolites with antimicrobial activity over other microorganisms present in the sample. *P. syringae* pv. *syringae* and *P. fuscovaginae* produce cyclic lipodepsipeptides (LDPs) as secondary metabolites, which act as antibiotics against a great number of gram-positive bacteria and fungi [48].

To compare pathogen detection in rice seeds by conventional PCR and BIO-PCR, artificially infected seeds were mixed with healthy ones at fixed proportions. At large pathogen amounts (10^8 c.f.u./mL), the use of macerated seed extracts led to optimal results (i.e., detection at one or more infected seeds/g) by both methods. This could be due to the outward release of the target bacteria from seeds after maceration. By this means, larger pathogen amounts were obtained supporting pathogen amplification even by conventional PCR, when the target bacteria was not enriched in culture media. This was in agreement with reports by Adorada *et al*. [49] on the effects of rice seed contamination and infection by *P. fuscovaginae* on rice establishment. They found that significantly more bacterial cells were released from crushed discoloured than from non-crushed seeds during total bacterial isolation, indicating that most seed-borne bacteria were located just on or under seed's hull [49].

As expected, detection capability of BIO-PCR and conventional PCR from macerated seeds decreased at smaller pathogen amounts (10^2 c.f.u./mL), and more infected seeds were needed to detect the pathogens (four or more seeds for *P. fuscovaginae* or *P. syringae*, and six or more for *B. glumae*). Furthermore, conventional PCR from non-macerated seeds were less effective either large or small pathogen amounts, probably due to the lower number of bacterial cells diluted in these extracts than when macerating seeds. Notably, conventional PCR required more infected seeds to detect the pathogens, as the enrichment step in the culture media is omitted.

Overall, these results indicated that the designed BIO-PCR assays can detect the target bacterial

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pathogens on or extracted from seeds, even at low infection rates. Conversely, this is not a natural assay and further investigations in naturally contaminated rice seeds are required. Other factors that might be involved in the applicability of this BIO-PCR must be further considered, such as primer efficiency in the presence of other plant DNA sources or natural contaminants of rice seeds.

Finally, BIO-PCR was evaluated in infected but symptomless plant material and compared with detection by conventional PCR. *P. fuscovaginae* and *B. glumae* were detected in 26 (87 %) and 18 (60 %) samples out of 30 symptomless ones. Those apparently healthy leaves probably harbored a bacterial population that was within the BIO-PCR threshold. Otherwise, such bacterial infection level was not large enough to induce symptoms or did not modified it nearby environment as to improve its habitat without causing the disease, while suppressing or evading plant defenses [50, 51]. On the other hand, the negative samples might have contained a population below the assays' detection limits. In this line, BIO-PCR has been used before as a reliable tool in pathogen detection in infected but symptomless samples. For instance, Shaad *et al*. detected *C. michiganensis* subsp. *sepedonicus* in 18 out of 22 symptomless but pathogen-suspect infected potato tubers using this technique [52].

In our case, Pss-BIO did not detected *P. syringae* in any of the tested samples, despite the high sensitivity shown. *P. syringae* pv. *syringae* strain B728a, which was used as positive control in this study, is a bean foliar pathogen that exhibits a very pronounced epiphytic phase in plants [53]. Nevertheless, it has been shown that a plant pathogenic bacterium can efficiently transmit from seeds to non-host seedlings by saprophytic multiplication, resulting in the establishment of a primary inoculum focus [54]. Particularly, the BIO-PCR tested plants showed a pathogen population bellow the detection limits, or there was not transmission of *P. syringae* pv. *syringae* from rice seeds to seedlings, with no detection by BIO-PCR in both cases. Further studies should address other seed inoculation strategies, such as vacuum infiltration, including foliar inoculation with subsequent detection in symptomless leaves.

Thus, conventional PCR did not amplify the three pathogens in any sample, even when DNA quality was checked using universal primers. This could be indicative of pathogen amounts bellow the thresholds in symptomless leaves, what can be solved with the previous enrichment step in BIO-PCR.

In summary, the three designed BIO-PCR assays offer greater sensitivity, detect only viable cells and at early infection phases of the diseases, playing an important role in the diagnosis of quarantine bacterial pathogens and for future epidemiological studies of bacterial diseases. These assays can reliably and efficiently diagnose *P. fuscovaginae*, *B. glumae*, and *P. syrinage* in rice seeds, guaranteeing that only bacterial-free propagating material could be sown and moved from one place to another. Advantageously, *P. fuscovaginae* and *B. glumae* can be detected in infected but symptomless plant material, supporting the application of effective control measures at early stages of disease development. Despite the promising results of BIO-PCR, more sensitive methods, such as real-time PCR and nested PCR, must still be considered, to prevent false negative results in asymptomatic plant propagating material.

Acknowledgements

This research was supported by a Short-Term Fellowship, awarded by the Arturo Falaschi Fellowship Programme of the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Received in October, 2022.
Accepted in December, 2022.