

Morphological, Biochemical and Molecular Characterization of the Causal Agent of Bacterial Panicle Blight Disease of Rice (BPB) In Selected Rice Production Zones of Zamfara State

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ABSTRACT

Bacterial panicle blight (BPB) is an emerging rice disease in Nigeria, causing up to 75% yield loss and widespread globally. In the 2019 season, rice farmers in Zamfara State's Gusau, Talata Mafara, Maradun, and Bakura communities reported significant yield declines due to an unidentified disease. Infected samples suggested BPB as the cause, yet the pathogen had not been identified in Nigeria. A trial was conducted to identify and characterize BPB pathogens in Zamfara State, aiming to screen rice germplasm and find resistant sources. Infected rice grains from the region were collected and cultured on King's B medium at 37°C for 48 hours in the microbiology lab of Federal University Gusau. The bacteria were purified to single colonies and analyzed morphologically and via polymerase chain reaction (PCR), identifying the strain as *Burkholderia glumae*. This strain produced toxoflavin, a compound linked to its pathogenicity. PCR amplification of a 1079-bp fragment using gyrB-specific primers confirmed *B. glumae* as the BPB agent in Nigeria. A pathogenicity test was performed on three commercial rice varieties (Faro 44, FARO 56, and FARO 59) using the syringe inoculation method at the Biological Garden, Department of Biological Sciences, Federal University Gusau. All inoculated rice varieties exhibited typical BPB symptoms within three days, similar to those seen in naturally infected rice panicles. This confirmed *B. glumae* as the causative pathogen and highlighted the urgent need for resistant rice varieties.

Keywords: Rice, Bacterial Panicle Blight, Zamfara, Pathogen, *Burkholderia Glumae*

1.0 INTRODUCTION

The occurrence of microbial diseases, including bacterial panicle blight (BPB) of rice, limits rice production globally. BPB, caused by *Burkholderia glumae*, has been reported in many rice-growing regions in Asia, Africa, North America, and Latin (Mirghasempour et al., 2018; Namgyu et al., 2020; Zhou, 2019). This disease can lead to yield losses of up to 75% and decrease milling quality (Kushwaha et al., 2020). In the 2019 rice-growing season, farmers in

Zamfara State experienced a decline in yields due to a bacterial disease with an unknown cause. Previously, factors like high temperatures and water stress were believed to cause panicle blighting (Mizobuchi et al., 2020). However, *Burkholderia glumae* has been identified as the causative agent of BPB in numerous rice-producing countries (Mulaw et al., 2018; Shew et al., 2019). This bacterium was first reported and described in Japan in 1956 as the causal agent of seedling blight and grain rot (Pedraza et al., 2018). Since then, it has become a serious threat to rice production worldwide. Toxoflavin, a phytotoxin produced by *B. glumae* has been reported to be its pathogenic determinant as well as an essential factor for its virulence. Thus, *B. glumae* strains that are not capable of toxoflavin production are virtually avirulent to rice plants (Valdez et al., , 2020). Symptoms of bacterial panicle blight include seedling blight, panicle blight, sheath rot, and grain discoloration with substantial yield losses (Nandakumar et al., 2009; Ramachandran et al., 2021; Shew et al., 2019). Preliminary visit to Farmers field revealed that the disease is new in Nigeria, no published report about its cause. Most of the commercially cultivated rice varieties in Zamfara State are susceptible to the disease. (Personal communication). As a result, it is imperative to characterize the causal agent of the disease. Several studies reported *B. glumae* is a seed-borne pathogen inferring that infected rice seed may serve as the primary source of inoculum for the disease (Faizal Azizi et al., 2023; Pinson et al., 2010). Besides, previous studies conducted using PCR analysis revealed that a considerable amount of both irrigation water and soil samples analyzed tested positive for *B. glumae* which indicates that, irrigation water and soil can also serve as a source of inoculum for BPB spread (Peñaloza et al., 2020).

Several management strategies were used in the control of the disease including; plant quarantine, use of chemicals, biological control, and use of genetic resistance (Faizal Azizi et al., 2023; Zhou, 2019).



Oxolinic acid is the only known chemical used in the control of BPB, however, this chemical is not available for use in many rice producing countries including Nigeria. More so, the use of oxolinic acid in Japan and some other countries where it is available for more than two decades has led to the unfortunate emergence of *B. glumae* strains that are resistant to Oxolinic acid. This limits its effectiveness as a bactericide in those countries (Ortega et al., 2020; Syahri et al., 2019). Besides, the use of 3-Phenylpropanoic acid (3-PPA), a secondary metabolite extracted from the genus *Enterobacter* was demonstrated to inhibit the growth of *B. glumae* strains, which may serve as a potential antibacterial compound for disease management (Suárez et al., 2019). Similarly, the use of *Bacillus strain* as a biocontrol agent in the treatment of rice seeds shows BPB infection reduction by up to 63.5% (Pedraza et al., 2018). Another effective approach to mitigate or control the occurrence of BPB is to use certified seeds that are free of the pathogen. Thus, this method requires inexpensive, rapid, and sensitive identification and quantification of *B. glumae* in certified seeds. To this end, identification and characterization of the causative agent of the BPB is a prerequisite to the management of the disease. Thus, morphological, physiological, and biochemical approaches have been used to identify and characterize plant-pathogenic bacteria (Ramachandran et al., 2021; Schaad, 2008). However, plant-pathogenic bacteria identification and characterization using the aforementioned methods may not be accurate and reliable because of high similarities among bacterial species of the same genus which may lead to false-positive identification (Faizal Azizi et al., 2023; Mulaw et al., 2018).

To ensure the accurate identification, more than one detection approach needs to be used for cross-checking. The use of a molecular method such as conventional polymerase chain reaction (PCR) and real-time polymerase chain reaction (RT-PCR) has shown to be highly sensitive and accurate in the identification and characterization of many plant-pathogenic bacteria including *B. glumae* (Fang et al., 2009; Kim et al., 2012; Saylor et al., 2006). Furthermore, the use of specific primers extracted from *gyrB* sequences and sequencing of the pathogen-specific gene should be capable of *B. glumae* detection and identification in rice samples (Lee et al., 2018). The objective of this research was to; (i) use culture media to isolate and identify the bacterial panicle blight (BPB) pathogen; ii) use PCR to verify the causative agent of BPB iii) carry out pathogenicity test on some commercial rice varieties .

2.0 MATERIALS AND METHODS

2.1 Sample Collection, Bacteria Isolation and Purification

Fifty (50) samples showing signs of bacterial panicle blight were collected from farmer's field across the three

local government area of Zamfara State. The infected grains were surface sterilized with 70 percent ethanol for 30 to 50s, then soaked in 1% sodium hypochlorite for 3 minutes, followed by rinsing with sterile distilled water three times. Samples were air-dried on a sterile filter paper in a laminar flow cabinet, after which they were transferred into a 10ml sterile bottle containing distilled water and crushed using a pipette tip. 100 µl of the suspension was dispensed and streaked on a Petri-dish containing King's B agar medium (20g protease peptone #3, 1.5g KH₂PO₄, 1.5g MgSO₄. 7 H₂O, 15 g Bacto-agar, and 15 ml glycerol) and the petri- dish was sealed and incubated at 37⁰ C for 48hours. The bacterial colonies isolated from cultured dishes were studied for their morphological characteristics relative to the reference strain of *B. glumae*. 25 ml cooled sterilized King's B medium (45⁰C), was poured into sterilized Petri-dishes (15 cm diameter) each. A loopful of the bacterium was streaked across the surface of the medium with a wire loop. Two more plates were streaked without recharging the wire loop with bacterial suspension. Petri-dishes were sealed, labeled, and incubated in an inverted position at 37⁰C. Bacterial developed single colonies within 2 days. The experiment was conducted in Microbiology Laboratory, Department of Microbiology, Federal University Gusau.

2.2. Biochemical Methods

Gram staining was carried out following the methods of Smith and Hussey (Smith and Hussey, 2005) . Briefly, a single colony from a 2-day old bacteria culture was placed on a clean slide. Using a sterile wire loop, 2 drops of water were added to the bacterial colony, and a smear was made. The heat was used to air-dry and fix the smear. Thereafter, the slide was flooded with crystal violet for 1 minute and rinsed gently in an indirect stream of tap water for 2 seconds. The slide was again flooded with iodine solution for 1 minute after which it was rinsed in a gentle and indirect stream of tap water for 2 seconds. After that, the slide was decolorized with alcohol until the decolorizing agent running from the slide runs clear. The slide was flooded with a counterstain, safranin for 30 seconds to 1 minute. After which the slide was rinsed in a gentle and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper. The results of the staining procedure were observed under oil immersion using a compound light microscope (Nikon Eclipse E200).The experiment was conducted in Biological Sciences Laboratory, Federal University Gusau.

2.2.1 Confirmation of the Gram Staining with Potassium Hydroxide (KOH) String Test

To further confirm the gram staining results, the KOH string test was conducted following the methods of Arti et al. 2003 (Arthi et al., 2003). Briefly, a drop of 3%



KOH was placed onto a glass slide after which a loopful of a 1-day old bacteria culture was emulsified in the KOH. The suspension was mixed continuously for about 60 seconds and by slowly lifting the loop, the formation of a string was observed. Gram-negative cells form a string within 60 seconds while Gram-positive cells are not affected.

2.3 MORPHOLOGICAL CHARACTERISTICS

2.3.1. Scanning Electron Microscopy (SEM)

With the help of a refrigerated centrifuge, an aliquot of 1.5ml bacterial culture was centrifuged at 13,000 rpm for 15 minutes. The pellet was washed with sterile physiological saline and fixed in 0.5 ml of 2.5% glutaraldehyde prepared in sterile saline at 4°C overnight. Thereafter, the pellet was washed continuously with saline and dehydrated using an acetone series of 70 -100%, then stored overnight in a desiccator. The particle was spread on SEM stubs, dried to a critical point in dried apparatus, platinum-coated, and examined using Scanning Electron Microscope. This experiment was conducted in the Central laboratory, Umaru Musa Yar'adua University, Katsina State.

2.4. VALIDATION OF THE CAUSAL PATHOGEN OF BACTERIAL PANICLE BLIGHT (BPB) WITH PCR

2.4.1 DNA Extraction

A single colony of the Bacteria was inoculated into 2 ml of the Luria-Bertani (LB) medium and incubated at 37°C at 170 rpm for 24 hours. Then, 1.5ml of this culture was taken to extract the genomic DNA by using a purification kit (Presto™ mini gDNA Bacterial kit) according to the manufacturer's instructions. NanoDrop 2000C UV-vis spectrophotometer (Thermo Scientific, MA, USA) was used to check the purity of the extracted DNA. The experiment was conducted in Microbiology Laboratory, Department of Microbiology, Federal University Gusau.

2.4.2. Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to validate the presence of the pathogen using specific primer BGgyrF (5'-GCAGCGGCAAGGAAGACG-3') and BGgyrR (5'-GTCGTCGCCCGACGTCTC-3') to amplify the DNA fragment. PCR amplification was initiated for 30 µl containing 2 µl of template DNA, 15 µl 2x exTEN PCR master mix, 1.5 µl of each primer, and 10 µl of nuclease-free water. Amplification was carried out on a Bio-Rad Thermal cycler T100™ with denaturation at 94°C for 1 minute, followed by 35 cycles at 94°C for 1 minute, 64.6°C for 1 minute, an extension for 72°C for 1 minute followed by 72°C of final extension for 5 minutes. Aliquots (7 µl) of each PCR product were loaded onto horizontal electrophoresis on

a 1.5% Tris-acetate-EDTA agarose gel (Promega) at 80 V for 60 minutes. Gels were stained with Syber safe for detection of 1079 bp for 16S and 317bp for the specific primer respectively. The fragment size of the PCR products was predicted using a 1-kb ladder (Invitrogen Co.). The experiment was conducted in the Biotechnology Laboratory, Department of Plant Science, Ahmadu Bello University Zaria.

2.5 Pathogenicity Test

Pathogenicity test was conducted on 3 Commercial rice varieties (Faro 44, FARO 56 and FARO 59) to test the virulence properties of the isolated pathogen. The rice varieties were sourced from Maslaha Seed Company limited. The experiment was conducted at the Biological Garden Department of Biological Sciences, Federal University Gusau.

2.5.1 Preparation of Seedlings and Planting

For each of the 3 varieties, 100 seeds were planted in seedling containers with sandy-loam soils for 10-15 days. The 15 days old seedlings were transplanted into plastic buckets (28 cm x 25cm) with sandy-loam soils at a distance of 3 seedlings per bucket. The seeds were first placed in the oven at 40°C for 8 hours to break dormancy if any, after which the seeds were soaked in petri dish plates filled with water and were allowed to stay for 24 hours. Thereafter, excess water was drained and the petri dish plates were covered and allowed for another 24 hours. The pre-germinating seeds were then transferred to the already prepared seed containers and allowed to grow for 10-15 days. The experiment was conducted in the Biological Garden, Department of Biological Sciences, Federal University Gusau for 4 months.

2.5.2 Preparation of Inoculum and Artificial Inoculation of *B. glumea* into rice plants

The Bacterial strain was grown in LB broth and was then incubated in an orbital shaker (Thermoline®) at 28°C for 16 hours and 150 rpm. Finally, two washes with 0.85% saline solution were performed and the microbial biomass was adjusted to a concentration of approximately OD₆₂₀=0.2 corresponding to 10⁸ CFU per ml measured with spectrophotometry as described by Mogi and Tsushima (Mogi and Tsushima, 1985). The pathogenicity test was performed using the artificial method of inoculation of *B. glumae* with a syringe, following the method described by Okuda and Wasano (Okuda and Wasano, 1994). The freshly prepared inoculum suspension was injected into the panicle bud, at the booting stage (60 days after planting) using a 1 ml sterilized needle (BD™ 23G1). Five panicles per plant were inoculated by injecting 0.2ml inoculum per panicle into the central part of a panicle using a syringe just before heading time. The inoculated plants were covered

with polythene bags to maintain the moisture for 24 hours after which the bags were removed (Figure 1). The disease development was evaluated at 7 days, 14 days, and 21 days, after inoculation. The experiment was conducted in the Biological Garden, Federal University Gusau, with daytime temperatures varying from 30°C to 37°C and > 80% RH under normal photoperiodic conditions.



Figure 1: Inoculated rice panicles covered with polythene bags for 24 hours to maintain moisture at the Biological garden Federal University Gusau

3.0 RESULTS AND DISCUSSION

3.1. Cultural Characteristic of the Isolated Pathogen

Early symptoms of bacterial panicle blight (BPB) caused by a pathogen were observed on rice panicles. Three (3) strains were isolated from BPB infected rice panicles showing dark brown discoloration from the Biological garden, Federal University Gusau. (Figure 2). Cultured plates showed similar morphological characteristics to the reference *B. glumae* strain on King's B media, having colonies that are yellowish, round, and swollen with a diffusible yellow pigment as shown in (Figure 3).



Figure 2: A closer look of infected panicles on rice variety FARO 44

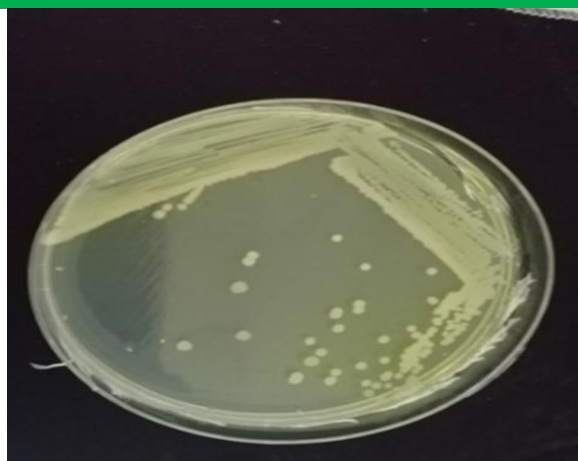


Figure 3: Bacterial colonies and production of yellow pigment (toxoflavin) on King's B agar. The photo was taken 48 hours after inoculation at 37°C

3.2 Biochemical Test

The bacterium gave a pink/red color after performing the gram staining procedure signifying that the isolated pathogen is gram-negative. To further confirm the gram stain result, the KOH string test was conducted and the bacterium was positive to the KOH string test.

3.3 Scanning Electron Microscopy (SEM)

In SEM, the bacterium was found to be a rod-shaped organism with cell-sized 1 to 5 µm in length and 0.5 to 1.0 µm wide. Singh and Vishunavat, (2015) observed that *Burkholderia* species are non-spore-forming, rod-shaped organisms with cell-sized 0.5 to 0.7 -1.5 to 2 µm. Flagella were also observed in the SEM image (Figure 4).

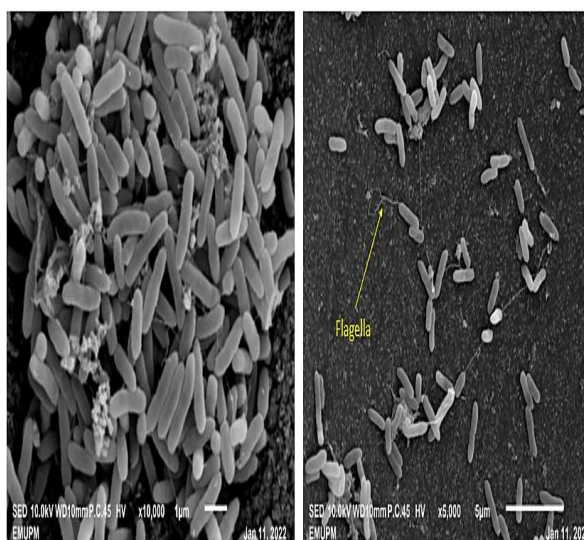


Figure 4: Rod-shaped structure of Bacterial strain viewed under the Scanning Electron Microscope (SEM)

3.4 Validation of the Causal Pathogen of Bacterial Panicle Blight (BPB) With PCR

3.4.1 DNA Extraction and Polymerase Chain Reaction

The identified bacteria isolates were further verified using specific primer BGgyr F (5'-GCAGCGGCAAGGAAGACG-3') and BGgyrR (5'-GTCGTCGCCCGACGTCTC-3') to amplify the DNA fragment. Approximately 317bp was amplified for gyrB specific primers as shown in Figure 5.

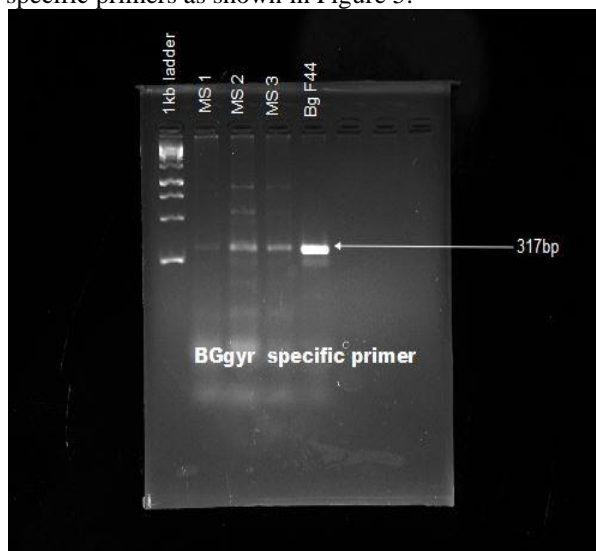


Figure 5: Gel photo of PCR amplification of 317-bp product for *B. glumae* with a pair of primers to detect specific DNA fragments corresponding to the gyrB nucleotide sequences, 1 kb ladder was used

3.5 Pathogenicity Test

3.5.1 Disease Incidence

The Isolated pathogen was confirmed to be pathogenic to all the 3 rice varieties under study. Visual symptoms on leaf sheaths and panicles were observed 3-5 days after inoculation until harvesting. The specific symptom of BPB observed is a blight on the panicles accompanied by the color changing. The infected panicles eventually have blighted kernels that first look white to light gray with a dark brown margin on the bottommost third part of the developing grains, which may be empty. Figure 6 A, B, and C typically illustrates the BPB symptoms on the panicle at the early growth stage, rachis branches stage, and harvesting stage.

Similarly, Figure 7. Shows the rice spikelets and their transformation from the pathogen attack, which changes the spikelets from normal green (Figure 7a) to reddish-brown (Figure 7 b). Subsequently, as the infection progresses, unfilled or aborted grains are observed (Figure 7 c). Figure 8. Shows a comparison between panicle inoculated with the pathogen (A) and panicle inoculated with sterile distilled water (B) two weeks after inoculation.

Bacterial panicle blight (BPB) is an emerging threat to global rice production, recently infesting many hectares in Zamfara State, Nigeria. To manage BPB effectively, it is crucial to identify its causal agent and screen rice germplasm for resistance. In this study, ten diseased panicles from commercial rice varieties were sampled and analyzed, confirming *Burkholderia glumae* as the pathogen.

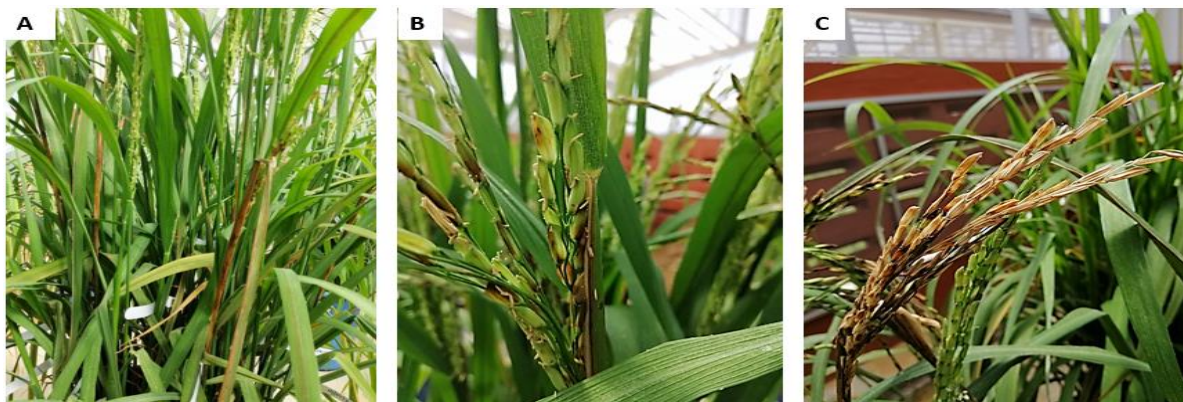


Figure.6: bacterial panicle blight symptoms on leaf sheath (A) early symptoms on panicles (B) at the early infection stage, the panicles turn reddish-brown (C) Infected kernels bear aborted seeds. The photo was taken 7 days, 14 days, and 21 days after inoculation.



Figure 7: Comparison between healthy kernels (A), the early infected kernels (B), and Kernels with aborted seeds (C) due to BPB infection

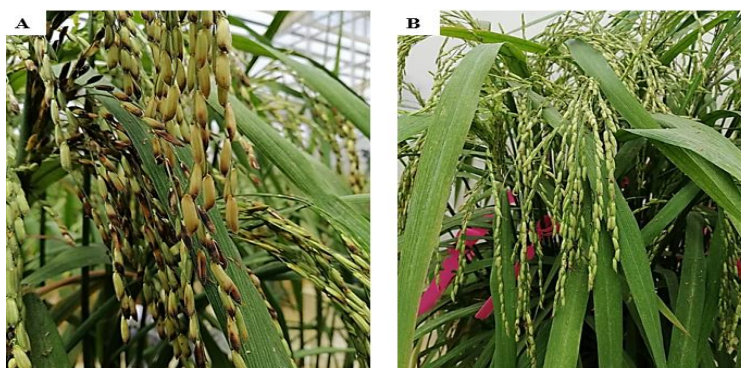


Figure.8: Comparison between panicle inoculated with *B.glumae* and (A) and panicle inoculated with sterile distilled water (B) Photo was taken two weeks after inoculation

3. 5. 2 Confirmation of Koch’s postulate

The microorganism found on the disease plant, when isolated and grown on, pure culture was obtained after a series of purification. When the cultured microorganism was inoculated into a healthy rice plant, the plant became diseased. When the microorganism was re-isolated from the diseased experimental host, it was identified as being identical to the original specific causative agent, thus completing the Koch’s postulates (Figure 9).

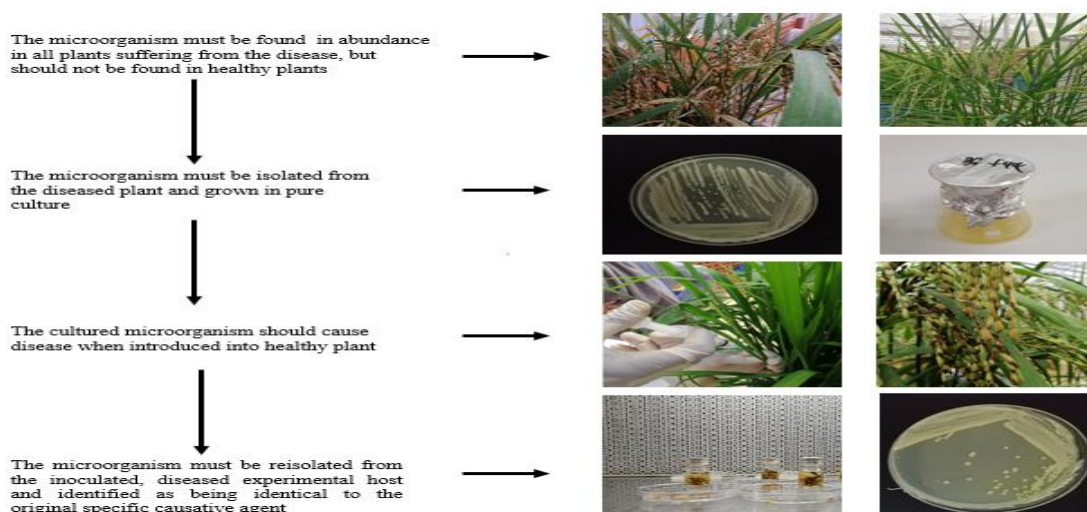


Figure 9: Confirmation of the Koch’s postulates by re- isolating from inoculated rice panicles and were grown on King’s B media to obtain a pure culture of the causal pathogen



4.0 CONCLUSION

In conclusion, due to the similarities across Burkholderia species, growth morphology and disease symptoms by themselves are not adequate to identify the primary pathogen of bacterial panicle blight (BPB) in Nigeria. Combining detection methods such as genetic, biochemical, morphological, and cultural approaches is necessary for accurate identification. This study used these techniques to define the BPB causal agent in Zamfara State and showed that the isolated strain was virulent against a number of commercial rice types. We verified Burkholderia glumae's identity as the BPB pathogen in Zamfara by completing Koch's postulate. The accuracy of selection in breeding programs can be maintained by timely inoculation and assessment, as demonstrated by the syringe inoculation method's reliable assessment of BPB disease resistance.

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