Journal of Applied and Natural Science 7 (2) : 562 – 566 (2015)

JANS

Identification of a seed-borne rice bacterium, *Burkholderia glumae* using cultural, morphological and biochemical methods

Deepali Singh and KarunaVishunavat

Department of Plant Pathology, G. B. Pant University of Agriculture and Technology, Pantnagar - 263145 Uttarakhand) INDIA
Corresponding author. E-mail: deepalisingh99@gmail.com

Received: January 20, 2015; Revised received: June 21, 2015; Accepted: July 15, 2015

Abstract: *Burkholderia glumae* is a seed-borne rice bacterium that causes bacterial panicle blight (BPB), which is a promising disease in many rice-growing areas around the world. The bacterium has been successfully grown on King's B agar medium (KBM) at 28^{0}C for 48 hrs. It is Gram negative, non-spore-forming, rod-shaped with cell size 0.5 to 0.7 - 1.5 to 2.0 µm with rounded ends. The colony appears as grayish white or yellow due to the pigment. The bacterium gave positive test in gelatin liquefaction, KOH solubility and nitrate reduction and while negative test in starch hydrolysis, oxidase reaction, levan production and arginine dihydrolase test. The bacterium utilized different carbon sources viz., sugars, amino acids, sugar alcohols and organic acids when examined through Biolog^{TM}GN2 Microplate System. This study would help in control and management of seed-borne bacterial plant pathogen *B. glumae*.

Keywords: Biochemical, *Burkholderia glumae,* Cultural, Morphological

INTRODUCTION

Burkholderia glumae is a bacterial plant pathogen that causes bacterial panicle blight in rice (*Oryza sativa*) Urakami *et al., (*1994). The bacterium may transmit through infected seed. On King's B agar Medium, the bacterium grows as circular grayish-white or yellow colonies at 28^{o}C (Coenye and Vandamme, 2003). It is non-sporulating, slow-growing and rod shaped bacterium with polar flagella. Tsushima *et al.,* 1986 reported relatively high temperature range (30–35°C) for the growth of *B. glumae.* Bacterial cultures could be identified using the Biolog^{TM}GN2 Microplate System (Jones, 1993). Biochemical tests indicate positive test for gelatin liquefaction, KOH solubility and nitrate reduction while negative test in starch hydrolysis, oxidase reaction, levan production and arginine dihydrolase test (Cottyn *et al.* 1996).Scanning electron microscopy is a well established technique for examining microorganisms. Overview of SEM theory and technique is given by Goldstein *et al.,*(1992). Since, this pathogen is getting prevalent in *tarai* region of Uttarakhand , India for the last 3 years, the present study was undertaken to isolate, the bacterial plant pathogen from infected rice-seed for identification through cultural, morphological and biochemical methods.

MATERIALS AND METHODS

Cultural characteristics

Isolation of bacteria: Rice plants showing characteristics symptoms of bacterial panicle blight were collected from Crop Research Centre and adjoining areas of G. B. Pant University of Agriculture and Pantnagar. Infected seed samples were sterilization with 1% sodium hypochlorite for2 minutes and successively washed three times in sterilized distilled water. These seeds were transferred aseptically, in a Petri-plates containing King's B agar medium. Bacterium was purified by serial dilution with sterile distilled water and plating on King's B agar medium.

Purification of bacterium: Pour sterilized King's B medium, cooled to about 45^{o}C, in sterilized Petri- plates (15 cm diameter) @ 25ml/plate. On solidify, these plates were inverted. A Streak was made through loopful of the bacterium, over the surface of medium in plates by to-and-fro motion of the inoculated needle. Two more plates were streaked without recharging the wire loop with bacterial suspension. Petri-plates were labeled and incubated in an inverted position at 28^{o}C. Bacterium develops colonies within 2 days. Single colonies are usually obtained in 2nd or 3rdPetri- plate and stored in refrigerator for further studies.

Effect of different temperature, media and incubation period on the growth of *B. glumae*: The experiment was carried out using three different media viz. King's B medium, Nutrient agar medium and Sucrose Peptone agar at four different temperatures of 25^{o}C, 28^{o}C, 30^{o}C and 35^{o}Cand three different incubation periods viz. 24 hrs, 48 hrs and 72 hrs. Each plate was poured with 25ml of the medium. A filter paper disc was dipped into the bacterial suspension and placed in the centre

of the each plate.

Effect of different category of seeds on recovery of *B. glumae*: The experiment was carried out using four different category of seeds viz. trace, mild, moderate and severe. The medium in each plate was poured @25ml/plate. Twenty five seeds of each category separately were placed in the Petri-plates and incubated at 28°C for 48 hrs. Each treatment was replicated three times and observations were taken after 48hrs.

Biochemical methods

Gram staining: Gram reaction was observed according to the procedure laid in the Bergey's Manual of Determinative Bacteriology (Buchanan and Gribbons, 1974).

Twenty four hour's old growth of culture was suspended in sterile water. Thin smear of the bacterial suspension was prepared on a glass slide and dried by passing over a flame. The dried smear is covered with Hacker's ammonium oxalate crystal violet stain for one minute and then washed in tap water. This slide is again flooded with Gram's iodine solution which is allowed to stand for one minute and washed further in tap water, blot dried and decolourised with alcohol until only faint violet colour remains in the solvent. In next step, the slide is again washed in tap water, blot dried and stained with safranine for 30 seconds, washed further in tap water, drained blot dried and examined under the microscope. Gram positive bacteria stain blue (violet) and gram negative red in colour.

KOH solubility test: On a glass slide, mix a loopful of bacteria from a well grown colony in a drop of 3% KOH aqueous solution. The mixing should not exceed 10s. A toothpick can also be used for picking bacterium from the colony and for mixing. While raising the loop from the glass slide, if strands of viscid material are seen, the bacterium is gram negative. Gram positive bacterium does not produce such strands even on repeated strokes.

Gelatin liquefaction: In this method, inoculum of an 18-24 hrs old colony of test bacterium is stab-inoculated into tubes containing Nutrient Gelatin medium. The inoculated tubes along with uninoculated control tube are incubated at 28°C for 1 week, checking everyday for gelatin liquefaction. Gelatin normally liquefies at 28°C or above. The inoculated tubes are then immersed in an ice bath for 15-30 minutes. Afterwards, tubes are tilted to observe if gelatin has been hydrolyzed. The medium in culture tubes with hydrolyzed gelatin will remain liquid medium even after exposure to cold temperature (ice bath) while the uninoculated control medium will remain solid. The hydrolysis of gelatin indicates the secretion of gelatinase by the test bacterium into the medium (Stolpe and Godkeri, 1981).

Nitrate reduction test: Nitrate broth medium (nutrient broth+ 5g/litre KNO_3) is prepared, poured in culture tubes and sterilized at 15 psi for 15 minutes. The cultural tubes containing NBM are inoculated with 48 h old pure culture of the bacterium and incubated at 24 -48 hrs 28°C. After incubation about 5 drops of a-naphthylamine and sulfanilic acid is added to the medium and shaked gently to mix the reagents. Positive reaction was identified by the formation of a pink or red color in the medium within 1-2 minutes following the addition of a-naphthylamine and sulfanilic acid or no color development within 5-10 minutes after adding zinc dust (Palleroni, 1984).

Biolog^{TM}GN2 Microplate System: This automated metabolic characterization system having 96-well microtiter plate arrayed with different carbon sources and the microorganism of interest. Biolog's system is based on the oxidation of carbon sources by organism of interest. Single colony of the bacterium was obtained from the culture grown on King's B Medium. The Bacterium was streaked onto Biolog Universal Growth Agar (BUG) medium. Approximate bacterial cells were quantified with a turbidimeter, and 150µL of this solution was poured into each of 96 wells in Biolog microplates. These microplates were incubated at 28^{0}C for 24-48 hrs. These Microplates were read for gram reaction, visually.

Morphological characteristics

Scanning Electron Microscopy (SEM): An aliquot of 1.5 ml of bacterial culture was centrifuged at 8000 x g in a refrigerated centrifuge 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 4ºC overnight. The pellet was washed repeatedly with saline and dehydration was done through an acetone series of 70-100% and kept overnight in a dessicator. The particle was spread on SEM stubs, dried to a critical point in dried apparatus, platinum coated and observed under Scanning Electron Microscope (Table 1).

RESULTS AND DISCUSSION

Cultural Characteristics: The bacterium could be most suitably grown on King's B agar medium (KBM) (20g protease peptone #3, 2.5gK_2HPO_4, 6.0g $MgSO_4$*$7H_2O$, 20 g Agar, and 15 ml glycerol) at 28°C for 48 hrs. *B. glumae* appeared as whitish grey or yellow colony on King's B medium due to yellow-green, water-soluble pigment. These findings are in accordance with (Coenye and Vandamme, 2003) who also reported grayish white or yellow colouration in the medium due to the water soluble pigments by *B. glumae* .

Effect of different temperatures, media and incubation periods on the growth of the bacterium: *B. glumae* when subjected on three different media, and four different temperatures for a period up to 72hrs for growth and multiplication, exhibited no growth on the Nutrient agar and Peptone sucrose agar media at any of the temperatures and incubation periods. On king's B medium bacterium exhibited the growth but at 25^{0}C, the bacterial growth was not observed even on KB

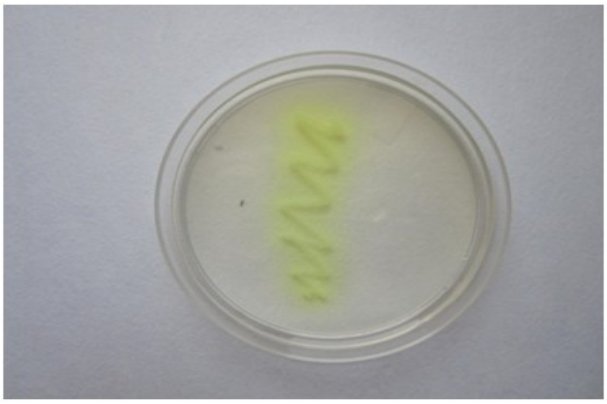

Fig. 1. *Growth of B. glumae on King's B medium*.

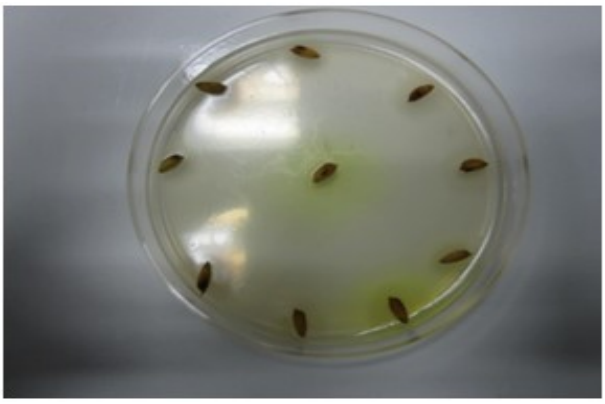

Fig. 2. *Recovery of B. glumae on different category of seeds.*

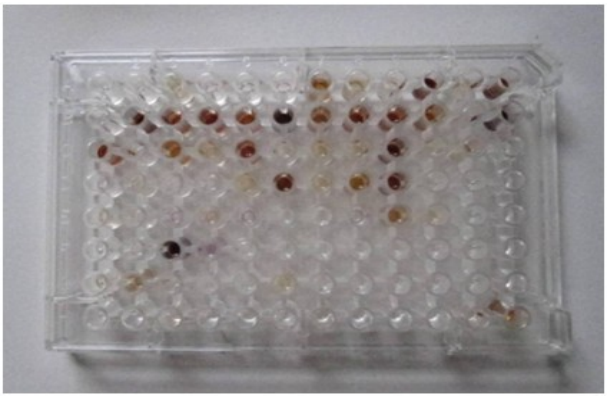

Fig. 3. *Utilization of different carbon sources by B. glumae***.**

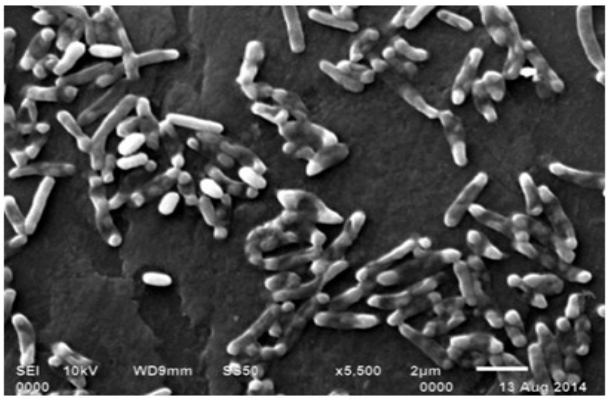

Fig. 4. *Rod shaped structure of B. glumae.*

Table 1. Evaluation of temperature, media and incubation period for growth of *B. glumae*.

Media	Temperature											
	25^0C			28^0C			30^0C			35^0C		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
King's B Agar Medium	-	-	-	-	++	+	-	+	+	-	+	+
Nutrient Agar Medium	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose Peptone Agar Medium	-	-	-	-	-	-	-	-	-	-	-	-

\+ indicates minimum growth, ++ indicates maximum growth, - indicates no growth

medium even after 72 hrs of inoculation. The minimal growth of bacterium was observed on King's B agar medium at 35^0C.The maximum bacterial growth was observed on King's B agar medium at 28^oC (Table 1).

Effect of different category of seeds on recovery of *B. glumae*: The experiment was carried out using four different category of seeds on the basis of seed colouration viz. trace, mild, moderate and severe. Table 2 shows that maximum percent recovery of bacterium was from severely infected (51.00%) seeds just after harvest. In order, the percent recovery of bacterium was recorded in moderately infected and mild infected seeds 12.30% and 8.33%, respectively. The minimum recovery of bacterium was in 1.1% and 1.0% in trace infected and apparently healthy seeds during 2013. Maximum percent recovery of bacterium was severly infected (54.00%) seed just after harvest. In order, the percent recovery of bacterium was recorded in moderately infected and mildly infected seeds 13.38% and 9.17%, respectively. The minimum percent recovery of bacterium was in trace infected and apparently healthy seeds 1.8% and 1.7% respectively during 2014.

Biochemical test: The bacterium gave positive test in gelatin liquefaction, KOH Solubility and nitrate reduction and while negative test in starch hydrolysis, oxidase reaction, levan production and arginine dihydrolase test

Table 2. Recovery of pathogen in different categories of seeds, immediately after harvest during 2013 and 2014.

Category of seeds	Recovery of pathogen (%)	
	2013	2014
Trace	1.1	1.8
Mild	8.33	9.17
Moderate	12.30	13.38
Severe	51.00	54.00
Apparently healthy	1.00	1.7
CD at 5%	2.90	2.41

Table 3. Biochemical characterstics of *B.glumae*.

Test	Result
Gelatin liquefaction	+
KOH	+
Nitrate reduction	+
Starch hydrolysis	–
Oxidase reduction	–
Levan production	–
Arginine dihydrolase	–

Table 4. Carbon utilization patterns using the Biolog GN2 MicroPlate system.

Carbon source utilization	*B. glumae*
14 sugars	L-Arabinose, , D-Cellobiose, D-Fructose, L-Fucose, D-Galactose, Gentiobiose, α-D-Glucose, α-D-Lactose, Maltose, D-Mannose, D-Melibiose, D-Psicose, Glucose-1-Phosphate, Glucose-6- Phosphate, D-Raffinose, L-Rhamnose, Sucrose , D-Trehalose, Turanose
4 sugar alcohols	D-Arabitol, m-Inositol, Adonitol, Xylitol
14 organic acids	Acetic Acid, Cis-Aconitic Acid, D-Galactonic Lactone, D-Galacturonic Acid, D -GlucosaminicAcid, D-Glucuronic Acid, p-HydroxyPhenylacetic Acid, Itaconic Acid, α-Keto Butyric Acid, α-KetoGlutaric Acid, α-KetoValeric Acid, Succinamic Acid, Methyl Pyruvate, Mono-Methyl- Succinate),
8 amino acids	L-Alaninamide, L-Histidine, Hydroxy-LProline, L-Proline, Uridine, Dextrin, N- Acetyl-D galactosamine, N-Acetyl-D glucosamine

(Table3). (Cottyn *et al.* 1996) reported that *B. glumae* shows positive test in gelatin liquefaction, KOH and nitrate reduction while negative test in starch hydrolysis, oxidase reaction, levan production, arginine dihydrolase test**.**

Biolog^{TM}GN2 Microplate System: Biolog^{TM}GN2 Microplate system is designed for identification of gram-negative bacteria. Out of 95 different carbon sources, as many as 45 were oxidized by *B. glumae*. These include 14 sugars, 4 sugar alcohols, 14 organic acids 8 amino acids (Table 4). Cottyn *et al*., 1996 is found biochemical methods such as Biolog for identify the bacterial pathogen.

Scanning Electron Microscopy (SEM): In SEM, the bacterium was found non-spore forming, rod-shaped organism with cell sized 0.5 to 0.7 - 1.5 to 2.0 µm with rounded ends. Holt *et al. (*1994) observed that *Burkholderia* species are aerobic, non-spore-forming, straight or slightly curved Gram negative rods that are 1 to 5 µm in length and 0.5 to 1.0µm wide.

Conclusion

B. glumae is found to be seed-borne non- spore forming, rod shaped, bacterium. For the detection of the bacterium from seed, King's B agar medium is found most suitable. The biochemical test of the bacterium exhibited positive test for gelatin liquefaction, KOH solubility and nitrate reduction while negative test in starch hydrolysis, oxidase reaction, levan production and arginine dihydrolase. The bacterium utilized different carbon sources viz., 14 sugars, 4sugar alcohols, 14organic acids and 8amino acids when examined through Biolog^{TM}GN2 Microplate System. In Scanning Electron Microscopy, the bacterium cell size is measured 0.5 to 0.7 - 1.5 to 2.0 µm with rounded ends. The cultural, morphological and biochemical characteristic of *B. glumae* would help in control and management of this seed-borne rice bacterium plant pathogen which is prevalent in *tarai* region of Uttarakhand, India for last three years.

REFERENCES

Buchanan RE, Gribbons NE (1974). Bergey'sManual of Determinative Bacteriology (8th edition).Williams & Wilkins Co. Baltimore USA.

Coenye, T. and Vandamme, P. 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches.*Environmental Microbiology*.5(9): 719-729.

Cottyn, B.; Cerez, M.T.; VanOutryve, M.F.;Barroga, J.; Swings, J. and Mew, T.W.1996. Bacterial diseases of rice. I. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. *Plant Dis*. 80 (4): 429-437.

Goldstein, J.I.; Newbury, D.E.; Echlin, P.; Joy, D.C.; Fiori, C. and Lifshin, E. 1992. Scanning electron microscopy and X-ray microanalysis; a text for biologists, materials scientists, and geologists.Plenum Press, New York.

Jones, J.B. 1993. Evaluation of the Biolog GN MicroPlate system for identification of some plant-pathogenic bacteria.*Plant Disease*. 77(6): 553-558.

Palleroni, N.J. (1984). Genus I. Pseudomonas Migula 1984, 237AL.In Bergey's Manual of Systematic Bacteriology, vol. 1, pp. 141–199.Edited by N.R. Krieg & J.G. Holt. Baltimore: Williams & Wilkins.

Stolpe and Godkeri. (1981). Non -pathogenic members of genus pseudomonas in the prokaryotes. Ed.Marthiner*et. al.,* Springer Verlag, New York, pp. 719 -741.

Tsushima, S.; Naito, H. and Koitabashi, M. (1995). Change in panicle susceptibility associated with flowering rate of spikelets in bacterial grain rot of rice caused by *Pseudomonas glumae*. *Annals of the Phytopath.Soc. of Jap*. 61 (2): 109-113.

Tsushima, S.; Wakimoto, S. and Mogi, S. (1986). Selective medium for detecting *Pseudomonas glumae* Jurita et Tabei, the causal bacterium of grain rot of rice. *Ann. Phytopath. Soc. Japn.* 52: 253-259.

Urakami, T.; Ito-yoshida, C.; Araki, H.; Kijima, T.; Suzuki, K.I. and Komagata, K. (1994). Transfer of *Pseudomonas plantarii* and *Pseudomonas glumae* to *Burkholderia* as *Burkholderia* spp. and description of *Burkholderiavandii* sp. nov. *International Journal of Systematic Bacteriology* 44:235-245.