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In-Soil or Residue Survival of *Burkholderia glumae*, Causal for Bacterial Panicle Blight in Rice

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Abstract

Bacterial Panicle Blight (BPB) is an important disease of rice worldwide. Based on our previous survey study, *Burkholderia glumae* was the main cause of BPB in Arkansas. Although there was no strong evidence *B. glumae* inhabiting soil or rice residue, rice producers in Arkansas were concerned about the pathogen's means of survival. To this concern, studies were designed to determine the survival and longevity of *B. glumae* in soil and rice residue. The studies included preliminary tests in laboratory and greenhouse to develop technique for bacterial survival field studies focused on soil and rice residue. Among the culture media tested (CPG, SMART, CCNT) and vegetable baits (celery, carrot, yellow onions), CCNT agar medium was best to recover and enumerate *B. glumae*. Both greenhouse and field tests showed *B. glumae* as short-lived in soil and residue suggesting the unlikelihood for it to overwinter and serve as an inoculum source to a new rice crop.

Keywords: Rice; *Burkholderia glumae*; Bacterial panicle blight; Survival in soil or rice residue

Introduction

Bacterial Panicle Blight (BPB) has a global distribution and is one of economically important rice diseases. It is mainly caused by *Burkholderia glumae* (syn.*Pseudomonas glumae*) and probably a few other species of *Burkholderia* including *Burkholderia gladioli* [1,2]. *B. glumae* was first described in Japan in the 1950s as the causal agent of grain rot and seedling blight in nursery rice. Since then, it has been reported in China, Korea, Latin America, Central America, South Africa and the United states [3-5]. Virulent strains of *B. glumae* produce a bright yellow phytotoxin on semi selective agar medium, known as toxoflavin as its major virulence factor [6-8]. Other virulent factors include formation of flagella [9], production of lipase [10] and catalase [11]. *B. glumae* follows a quorum sensing system in toxoflavin production and catalase expression [12]. In a quorum sensing, a cell-to-cell communication allows bacterial cells to collectively behave like a multicellular organism [13].

In severe disease years, BPB ranked among the three most yield limiting rice diseases in southern United States [14]. Arkansas produces nearly 50 percent of the rice grown in the U.S.A which makes it the number one rice producer in the nation. Arkansas rice contributes more than $4 billion dollars into Arkansas's economy every year (Farm Bureau Arkansas). In 2015 and 2016 a survey conducted on different rice growing counties in Arkansas indicated that *B. glumae* was the major cause of BPB in Arkansas rice fields [7]. BPB epidemics of year 2010 and 2011 with unexpectedly high grain yield losses led to apprehension on understanding the pathogen's means of survival. Of more concern was for fields having a severe BPB epidemic with zero-grade slope and continuous rice production year after year. Conventional practice in AR rice production uses alternating rotation with soybean. During the epidemic years of 2010 and 2011, there was no strong evidence that favored *B. glumae* to inhabit soil or rice residue. In the U.S.A. rice seeds are either drilled or water seeded. The early season damages from herbicides and weather conditions generally make diagnosis from biotic factors difficult. Subsequently obvious signs of rot or blight due to this bacterium at the vegetative growth stage has not been detected in the U.S.A. Therefore, this survival study was initiated to evaluate the longevity of *B. glumae* in soil and residue during the off season of Arkansas commercial rice production that typically extends from early October to mid-March [15,16].

Materials and Methods

Studies on technique development

Laboratory study on development of technique to isolate *B. glumae* from soil: To determine the best technique that detects *B. glumae* from low concentration in the soil, comparative tests were carried out between culture media and vegetable baits. CCNT medium [17] CPG medium and SMART medium [18] were selected based on earlier studies in our laboratory and a literature search. Yellow onion, carrot and celery were selected as baits based on literature and availability [19]. A 15 ml bacterial suspension approximately 4 x 10^4 CFUs/ml (colony forming units/ml) was prepared in a salt-sugar buffer [20] and applied to 50 gm sterilized or non-sterilized soil that were handled separately. A gram of each soil was thoroughly suspended in 10 ml of sterilized water. A 0.1 ml aliquot of soil suspension was transferred to petri dishes containing CCNT medium for each in two replications to serve as control. Then, a 1:1 serial dilution of each soil suspension was carried out 15 times. A 0.1 ml from each dilution was plated in duplicate on the three selected agar media and was incubated for 48 hours at 39°C. Colony forming units of *B. glumae* were counted based on characteristic cultural morphology using the colony morphology of the original isolate as a reference.

In Figure 1 the yellow onion scales or cross sectioned pieces of celery and carrot were surface sterilized with 0.05% sodium hypochlorite solution and rinsed in sterile water. The sterilized pieces were scratched with sterile scalpel to create a wound and each was placed on moist filter paper in a petri dish. Then they were inoculated with 10 µl undiluted stock suspension made from sterile soil. Vegetable baits were incubated at 30°C for 7 days to note if sunken water soaked lesions formed. Later tissue from the lesion was streaked onto CCNT medium to confirm the identity of the bacteria recovered using morphological characteristics. The CCNT agar medium is a semi-selective medium containing 2 g of yeast extract, 1 g of

polypepton, 4 g of inositol, 10 mg of cetrimide, 10 mg of chloramphenicol, 1 mg of novobiocin, 100 mg of chlorotharonil and 18 g of agar in 1000 ml of distilled water, and adjusted to pH 4.8 [17].

Figure 1: Surface sterilized pieces of carrot, celery and yellow onion scale placed on moist filter paper in a petri dish, scratched with sterile scalpel to create a wound to bait *B. glumae* from artificially infested soil (Top) and growing a scoop using needle plated on CCNT to confirm identity (bottom two left) in contrast to the original isolate (bottom one right).

Greenhouse study on in-soil survival of *B. glumae***:** To investigate duration of the field sampling of *B. glumae* in-soil, a preliminary greenhouse test was conducted. A kg of silt-loam soil sample was collected from Rice Research Extension Center (RREC), near Stuttgart, Arkansas. The soil was pre-moistened before autoclaving for one hour on a solid cycle, and then allowed to cool overnight. Plastic pots (~ 10 cm diam.) were disinfected with 10% sodium hypochlorite, rinsed with sterile water then air-dried before filled with soil to make four pots. A 48 hour *B. glumae* culture was harvested from King's B medium and transferred in to a salt-sugar buffer to obtain a bacterial suspension. Each of three pots received 5 ml of approx. 6.9 x 10^9 CFUs/ml of *B. glumae* suspension and thoroughly mixed using a gloved hand. Inoculated pots were then placed in separate plastic bags to collect water leachate during the experiment. The control pot was handled similarly except it received 5 ml sterile water. Pots were watered intermittently with sterile distilled water allowing alternate wetting and drying to simulate field conditions.

Soil from each pot was sampled at the beginning of the experiment and continued monthly for 5 months. Samples were obtained by gently pushing a plastic-drinking-straw (~ 0.5 mm dia.) down into the soil. Nearly 0.5 g of soil was collected along the depth of each pot. To represent the top and bottom soil profiles in the pot, the straw filled with soil was cut in half using sterile blade. Soil was carefully removed from straw pieces and vortexed separately with 5 ml sterile water. A 100 µl aliquot of suspension was spread onto each of two CCNT plates and incubated at 39°C. To serve as reference for colony morphology, the pure isolate of *B. glumae* that was used to inoculate/ infest the soil was plated on CCNT medium and incubated together at the same time. From each plate, CFUs that showed similar cultural morphology to the known *B. glumae* were counted starting from 48 hours from incubation until 96 hours. To test the leachate for *B. glumae*, 0.1 ml leachate water from each pot was tested by streaking on CCNT medium in three replications. The plates were kept in an incubator at the temperature described above and colonies were counted similarly.

In-field soil survival study of *B. glumae:* Once the methods of *B. glumae* detection were established from the laboratory and greenhouse pilot studies described above, a field study was carried out from the fall season of 2015 to the winter of 2018. A silt-loam soil was collected using well-washed buckets from RREC corn field known to have no rice grown in the preceding 5 years. Primarily, the soil was tested on CCNT agar medium for the absence of *B. glumae*. Three batches of nearly two kg soil were air-dried and pulverized to create homogenous mixtures. Two 600 ml bacterial suspensions of *B. glumae* were prepared as described for greenhouse study with concentrations of approximately 6.9 x 10^9 and 5.5 x 10^8 CFU/ml of soil suspension along with 600 ml sterile water for control. Each liquid was added to a separate batch of soil and thoroughly mixed using a gloved hand. Each soil batch was then sub-divided into 200 g samples and shaped to form one of 10 soil columns that were individually wrapped with nylon mesh and positioned as treatments to be placed on soil surface or buried at about 15 cm depth in the field from where the soil sample was collected. A zero-week sub-sample was processed for each soil batch to determine the initial cfu/g of soil. Then the labeled columns were placed accordingly under a secured covering with an iron mesh of 7 m^2 for the duration of study. Soil bundles from each treatment were removed monthly and brought to the laboratory to recover *B. glumae* on CCNT agar medium. Due to the lack of bacterial recovery on the first month of the study, the sampling protocol was modified to include a 2-week sampling following the initial day for the 2nd study. To recover and enumerate *B. glumae*, 1 g soil was mixed in 10 ml sterile distilled water in a culture tube. The soil suspension was vortexes for 5 sec and 1 ml aliquot was removed to create a series of 1:10 dilutions. From each dilution, 100 µl was streaked on two CCNT agar medium plates. For morphology reference, additional plates were cultured on CCNT with a suspension of *B. glumae* used to inoculate the soil samples. Plates were placed in an incubator set at 39°C. Recovered colonies were counted starting 48 hours after incubation and continued until 96 hours.

In-residue survival of *B. glumae* **under field conditions:** Since BPB symptoms are not common on rice sheath, stem or leaf; artificially inoculated panicles were used for this study. A cluster of rice panicles with classic symptoms of bacterial panicle blight (Figure 2) were selected from artificially inoculated rice nursery at RREC.

Figure 2: Bacterial panicle blight in rice mainly affects maturing grains resulting in either destroying embryos or producing light weight seeds and hence causing severe yield loss.

The nursery was artificially inoculated by spraying a suspension of *B. glumae* at approx 1.0 x 10^9 cfu/ml at anthesis. Panicles were

collected on the 2nd week of October towards the end of harvesting time in Arkansas. Tests to recover *B. glumae* were carried on every month until the 2nd week of March that often marks the beginning of early rice planting. Ten bundles each with five panicles were randomly selected. Twenty kernels were randomly picked across each panicle to obtain 100 kernels/florets per bundle. Ten kernels were embedded in a CCNT agar per petri dish. Plates were incubated at 39°C for up to 96 hours. Control plates were plated on CCNT culture medium with a blend of three *B. glumae* isolates that were used to inoculate rice florets in the field. Kernels surrounded with a yellow toxoflavin pigment of *B. glumae* were marked and counted as positive from 48 hours to 96 hours of incubation indicating the survival and multiplication of the bacteria. After the initial sampling, each sub-sample of panicles was carefully wrapped, secured in nylon mesh, and tagged as either on soil surface or buried at 15 cm. The wraps were placed in a cornfield that was not cultivated with rice for at least the preceding five years. The plot area was secured with 7 m^2 iron mesh placed as cover. A bundle of panicles from each treatment was removed monthly and brought to the laboratory to test kernels for *B. glumae* on CCNT agar medium. In the absence of kernels, glumes/ chaffs were plated. On the same day the test started, kernels from initial sub-samples were placed on CCNT to determine the number of kernels/florets positive for *B. glumae*. The experiment was carried out for three off seasons starting year 2015 through the winter of 2018.

Results

Studies on technique development

Laboratory study on development of technique to isolate *B. glumae* **from soil:** Use of CPG agar medium and CCNT provided recovery of *B. glumae* from different dilutions using a gram of inoculated soil in 48 hours after incubation at 39°C. However, CPG allowed more contaminates to grow especially at lower dilutions of soil. SMART agar medium failed to show any colony growth in 48 hours.

It took nearly a week before distinctive bluish colonies appeared on SMART medium. Due to contaminants in CPG and lengthy incubation period with SMART medium, CCNT allowed production of a yellow translucent color and was chosen as the best medium with recovery of bacterium at ~ 1.0 x 10^6 CFU/ml and 1.0 x 10^7 CFU/g soil.

There was considerable variation with the vegetable baits to successfully recover *B. glumae* from soil. Yellow onion scales detected the bacterium at a 3-fold dilution, celery a 2-fold dilution, and carrot at 1-fold dilution. Use of baits were not continued in this study as all were less sensitive in detecting *B. glumae* than the CCNT agar and required CCNT to confirm the presence of *B. glumae* with the lesion development in the baits.

Greenhouse study on in-soil survival of *B. glumae***:** *B. glumae* survived in greenhouse soil for up to four months with a substantial decrease in population size overtime. The greenhouse temperature ranged from 25.6°C to 28.3°C during the test period. *B. glumae* survived longer in the bottom and wetter part of the sterilized field soil than on the soil surface that dried out with irregular watering. *B. glumae* was not recovered from the leachate beyond the initial sample (Table 1).

Soil Depth in a Pot	Sampling Month					
	October/Initial*	November	December	January	February	March
Top	+++	++	-	+	-	-
Bottom	+++	+	+	+	+	-
Leachate	++	-	-	-	-	-

Table 1: Comparison of survival of *B. glumae* in the bottom, surface soils and leachate in a greenhouse pot test.*Bacterial smear on a plate were indicated with more than one positive sign depending the smear amount while a few separated colonies with a single positive sign. The negative sign indicated no recovery of *B. glumae*.

Field studies

In-soil survival test of *B. glumae* **under field conditions:** Like the pilot study in the greenhouse, this study also showed *B. glumae* to be short-lived regardless of the high initial CFUs/g of the artificially infested soils.

In 2015, no colonies of *B. glumae* were recovered from the soil in samples tested a month after the infested soils were placed on soil surface or buried at 15 cm depth in the field plots to simulate no till or tilled soils of Arkansas rice farms. Due to the absence of live *B. glumae* recovered from first month test in year 2015, a 2-wk sampling time was then included in the following years of this study. Unlike the greenhouse test, no CFU of *B. glumae* was recovered from the buried samples in year 2016 whereas there was recovery from "on soil surface" samples with sharp decline in *B. glumae* population. In successive tests from November through March of year 2016, *B. glumae* was not recovered from both "surface" and "buried" samples. In 2017, *B. glumae* was recovered in the 2-wks tests both from "surface" and "buried" samples with nearly 50% decline in CFUs compared with the initial samples (Table 2).

Year	Sample Position	*B. glumae* Density	November CFU/g Soil[x]	2wk After CFU/g Soil[y]	CFU/g Soil until March
2015	Surface	High	6.9×10^9	NA[z]	0
		Low	5.3×10^8	NA	0
	Buried	High	6.9×10^9	NA	0
		Low	5.3×10^8	NA	0
	Control	Uninoculated	0	NA	0
2016	Surface	High	3.6×10^6	2×10^5	0
		Low	1.7×10^6	7×10^3	0
	Buried	High	3.6×10^6	0	0
		Low	1.7×10^6	0	0
	Control	Uninoculated	0	0	0
2017	Surface	High	3.6×10^6	0	0
		Low	1.7×10^6	0	0
	Buried	High	3.6×10^6	2.7×10^3	0
		Low	1.7×10^6	2.0×10^3	0
	Control	Uninoculated	0	0	0

Table 2: Colony Forming Units (CFUs) of *Burkholderia glumae* recovered from a gram of soil artificially infested with bacterial suspension at 6.9×10^9 or 5.3×10^8 CFUs and placed on the surface and buried at 15 cm depth in a field plot to evaluate survival through October to March in 2015, 2016, and 2017. X: The differences in initial *B. glumae* population recovered may be due to soil sample sources in the respective years. Y: The two weeks test was included in 2016 and 2107 since no *B. glumae* was detected in 2015 from the November samples. Z: NA= not available. Years show starting times. However sample tests continued until March of the following year.

In-residue survival of *B. glumae* under field conditions: Positive florets to *B. glumae* in the initial samples ranged from 24% to 35%, 17% to 29%, and 18% to 40% in year 2015, 2016 and 2017, respectively (Table 3).

Percentages of *B. glumae* Positive Florets across Time									
Year	**Panicle Position**	**Infected with *B. glumae***	**Sampling Time**	**2 wk**	**Nov**	**Dec**	**Jan[y]**	**Feb[y]**	**Mar[y]**
2015	Surface	Yes	Initial	NA[x]	28	26	26	32	30
			Later		0	0	0	0	0
	Buried	Yes	Initial	NA	35	27	31	33	24
			Later		0	0	0	0	0
2016	Surface	Yes	Initial	20	19	16	19	18	20
			Later	0	0	0	0	0	0
	Buried	Yes	Initial	24	26	20	26	29	17
			Later	0	2	2	0	0	0
2017	Surface	Yes	Initial	32	27	36	18	40	NA
			Later	15	16	13	6	0	NA
	Buried	Yes	Initial	25	30	23	19	34	NA
			Later	22	10	3	0	0	NA

Table 3: Percentages of initial infected seeds recovered as positive to *B. glumae* compared to positive seeds/florets recovered after "on surface" and "buried at 15 cm depth" treatments in a field plot across 5 months in 2015, 2016 and 2017. X NA=Either samples were not available or not tested due to the zero result in prior months. Y: Years show starting times. But ample tests continued until March of the following year.

Both surface and buried treatments showed no *B. glumae* recovered from kernel/floret from field plots in 2015. As a result, a 2-wk sampling interval was added in the latter two years of the study. For the entire sample period in 2016, no positive florets were recovered from field plots for "surface" treatment. In the case of the "buried" treatment, a couple of hits were detected for November and December only. In the 3rd year of the study, positive kernel/floret was obtained until December from the "buried" samples and until January from samples placed on "soil surface". No recovery of *B. glumae* occurred in February for kernel/florets collected regardless of sample placement in the field (Table 2). No differences in decomposition pattern were observed for the residues when buried in soil or left on the soil surface.

Discussion

Our previous survey study indicated that *B. glumae* was the common bacterium that causes BPB in Arkansas. Based on this result, preliminary laboratory and greenhouse tests were conducted to develop technique to subsequently conduct a survival study in soil and residue under field conditions. In the laboratory preliminary tests, when CCNT, CPG and the SMART agar media were compared, CCNT provided recovery of *B. glumae* from different dilutions of *B. glumae* in artificially infested soils. Among the vegetable baits (yellow onion, celery and carrot), the scales yellow onion allowed *B. glumae* to grow better forming distinct sunken lesions. However, the semi-selective medium, CCNT was required to ultimately confirm the identity of the bacterium that grew on yellow onion scales. Therefore, CCNT was selected as the sole preferred method to pursue the field in-soil and residue survival study. No PCR (polymerase chain reaction) or other molecular tests were considered to detect DNA segments since the intent was to recover viable *B. glumae*.

Different agricultural management practices such as tillage, rotation, and burning can lead to changes in soil structure which can increase or decrease soil-borne plant diseases. For instance, incorporating organic amendments and/or crop residue has a direct impact on plant health and crop productivity depending on an amount and quality of organic matter that is returned to the soil. Therefore, pathogen viability, distribution, nutrient availability, and release of biologically active substances can be influenced [21]. In some other instances, conservation tillage is placed to conserve soil moisture. Such a practice leaves crop residue on the soil surface after harvest. As a result, crop residues may also provide food and act as a habitat for certain plant pathogens [22]. Unlike *Xanthomonas campestris cv. campestris* populations fluctuating in time and survival in Brussels residue till after 24 months [23], the field test results of our study indicated that *B. glumae* to be poorly adapted to soil or residue environment. In addition, there are no reports of *B. glumae* infection on the rotation crops used in Arkansas rice production systems. Although there was no strong evidence against *B. glumae* inhabiting soil or rice residue, it was important to clear the doubts Arkansas rice producers had as to the source of inoculum to their new crops.

B. glumae is known to cause severe wilt symptoms in tomato, hot pepper, eggplant, potato, perilla, sesame, and sunflower reported in 2000 and 2001 in Korea. However, there are no reports of other hosts to *B. glumae* in the U.S.A. Our study did not cover the survival study of *B. glumae* in other live host plants. Moreover, our study did not investigate in-soil or residue survival of the other species of *Burkholderia* such as *B. gladioli* that are known to cause similar panicle blight in rice. With such gaps of information, it is difficult to definitively conclude that there can be no chance of *B. glumae* or other species of *Burkholderia* to cause panicle blight in rice production system in Arkansas.

However, our study supports that *B. glumae* as short lived in soil environment. *B. glumae* is a pectolytic, gram-negative, capsulated, motile, with *lophotrichous* flagellated bacterium but it is not spore producing to adapt to be carried over long unfavorable periods [24]. Most agree with the idea that as in most plant pathogenic bacteria that infect annual agricultural plants, *B. glumae* survives from season to season in seeds rather than soil, residue or other host plants [25-27]. The sudden severe bacterial panicle blight situations in year 2010 and 2011 in Arkansas and other southern rice producing states of the U.S.A. may have been largely caused by the *B. glumae* and probably other one or more species of *Burkholderia* that inhabited seeds. As an additional support that *B. glumae* is largely seedborne, our test in ratoon rice showed that the number of infected seeds sharply dropped in ratoon compared to the main crop.

BPB has been observed causing high yield loss under extended hot night temperature often above 24°C particularly around flowering stages. It seemed not to be favored by dry conditions. Studies by Wamishe et al. showed symptoms to be more pronounced under moist conditions such as under dew, mist, rain or windy rain and where dew stays longer such as east-side tree lines in a rice field. No wide outbreaks of BPB have occurred in year 2012 when the weather condition was hot and dry. Had the bacterium survived in soil or residue for a long time, the wet seasons of 2013 and the successive rice seasons could have been ideal for another outbreak. Our study greatly suggested the unlikelihood of the bacterium to over-season in soil or residue to infect new rice plants. To our knowledge this is the first report on survival study of *B. glumae* that cause bacterial panicle blight in rice under field conditions.

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