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Research Paper

Suppression of Bacterial Leaf Blight(Xanthomonas oryzae pv. oryzae) using Local Isolates of Rhizobacteria

Fedencio V. Paslon Jr.[A], Revelieta B. Alovera[B], Carolina D. Amper[B], & Myrna G. Ballentes[C],
[A]Biological Stress Management Department, R&D, Syngenta Philippines Inc, General Santos City, 9500 Philippines,
[B]Department of Plant Pathology, College of Agriculture, General Santos City, 9500 Philippines,
[C]Department of Entomology, College of Agriculture, Central Mindanao University, Musuan, Maramag, Bukidnon, 8710 Philippines

ABSTRACT

Local isolates of rhizobacteria associated with rice rhizosphere were evaluated against *Xanthomonas oryzae pv. oryzae* (Xoo) both *in vitro* and *in vivo* conditions. Twenty-three rhizobacteria were isolated from the rice-growing provinces of Caraga Region, Philippines, and their antagonistic activity were evaluated against Xoo causing bacterial leaf blight (BLB) on rice using well diffusion assay method. Among the isolates evaluated, three (FVP 08, FVP 09, and FVP 22) were found highly antagonistic against the test pathogen with a clear and strong degree of the zone of inhibition. The three rhizobacterial antagonists were further evaluated for biological control against Xoo under greenhouse condition. The application of these rhizobacterial antagonists on inoculated rice plants showed shorter lesion length, effectively suppressed the bacterial leaf blight and managed to reduce the severity of BLB.

Keywords: antagonistic effect, biological control agents, rhizospheric bacteria, rice
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INTRODUCTION

Rice crop is susceptible to a large number of diseases (Muneer, Rafi, & Akhtar, 2007, p.743) and among the most destructive bacterial diseases of rice belong to the genus *Xanthomonas*, which mostly comprises phytopathogenic bacteria, is a member of the family Pseudomonaceae. Among the xanthomonads, *Xanthomonas oryzae* pv. *oryzae* causes bacterial blight of rice is one of the most important diseases of rice in most of the rice growing countries (Hopkins, White, Choi, Guo & Leach, 1992, p. 451). It is found worldwide and is particularly destructive and epidemic potential in Asia to high-yielding cultivars during the heavy rains of the monsoon season in both temperate and tropical regions especially in Asia (JIRCAS, 2006)

Synthetic antibacterial chemicals mostly copper based, although often effective, are a significant expense for rice farmers. Their use, however, has many drawbacks and serious effects including destruction of beneficial micro-flora, an excessive level of unmonitored chemical residues to the crop, environmental contamination, health hazards, development of resistance of the bacterium to active compounds, and the potential to high phytotoxicity (Janse, 2005). It is, therefore, appropriate that an alternative and compatible measure for bacterial blight disease be introduced and applied.

The promising performances of the local rhizobacteria isolates have a great potential in the future to reduce the damage due to bacterial blight (Velusamy, Immanuel, Gnanamanickam, & Thomashow, 2006, p. 56). The recent dis-

**Corresponding Author: Fedencio V. Paslon Jr. E-mail: fedencio_jr.paslon@syngenta.com

coveries of Thangavelu and Mustafa (2012) on different isolates of rhizobacteria showed great antagonistic potential to bacterial leaf blight disease and have the capability to protect and promote plant growth by colonizing and multiplying in both rhizosphere and plant system.

This study was undertaken to assess the antagonistic capacity of local rhizobacteria applied as a seed treatment, seedling root-dipped and foliar spray against *Xanthomonas oryzae* pv. *oryzae*.

METHODOLOGY

Isolation and Purification of the Pathogen

Diseased samples of rice at panicle initiation stage showing typical bacterial blight symptoms were collected from Philippine Rice Research Institute- Agusan Experimental Station. Diseased leaf sections (2x7 mm) were surface sterilized with 1% sodium hypochlorite solutions then rinsed thoroughly with sterile distilled water. These sections were transferred into the sterilized distilled water in test tubes to allow bacterial oozing. A loopful of the suspension was streaked on previously plated peptone sucrose agar (PSA) and was incubated at room condition for 24 to 48 hours. Yellow, mucoid, dome-shaped colonies with entire margins that grow on the culture plates were further sub-cultured.

Isolation of the Potential Rhizobacteria

Figure 1 illustrates that the potential rhizobacterial antagonists that were sampled from different rice-growing provinces in Caraga Region, Philippines, namely: Agusan del Norte, Agusan del Sur, Socorro Islands, Surigao del Norte, and Surigao del Sur. About 1 gram of the rice soil rhizosphere was suspended in 10 mL sterile distilled water. The suspension was shaken using a thermo rotator at 150 rpm for 30 minutes and was heated at 800^{O}C water bath for 30 minutes. A serial dilution was prepared by adding 1 mL of suspension to 9 mL sterile distilled water until at 10^{6} dilutions were obtained and 0.1 mL of sample was spread on PSA plates and incubated at 30°C for 24 hrs. The growth of colonies was further subcultured and purified using the same medium (Beric et al., 2012).

In vitro Screening of the Potential Rhizobacteria

Rhizobacterial isolates were screened against Xoo using the well diffusion assay. Pure cultures of the differenct isolates were grown in a liquid containing peptone and sucrose for 48 hours.

Figure 1. Map of Caraga Region showing the Rice-growing Provinces where the Antagonist Rhizobacteria were Isolated

In a separate plated medium, Xoo suspensions (approx. 10^{6}cfu/mL) were seeded uniformly using pour plate method. Five equidistant wells were made in the previously plated medium seeded with Xoo by using a sterilized cork borer and using a sterile syringe of a 0.1 mL of rhizobacteria (10^{8}cfu/mL) was placed on the wells. The assayed plates were incubated for 24 to 48 hours and the zones of growth inhibition of Xoo around the rhizobacterial antagonists were measured (Beric et al., 2012).

In vivo Screening of the Potential Rhizobacteria

Different isolates of the rhizobacteria that inhibit the growth of Xoo under *in vitro* test were separately applied to rice seeds of NSIC Rc224. The seeds were soaked in 0.01% polyoxyethylene –sorbitan monolaurate (Tween20, Sigma Aldrich) containing 10^{8}cfu/mL of the rhi-

zobacterial antagonist, and then kept at 28 °C for 48 hours. The control seeds (without rhizobacteria) were soaked in sterile water. Subsequently, both control and treated seeds were sown in seedbeds using natural field soil. Seedlings were transplanted into plastic pots after 21 days. At the time of transplanting, the seedlings were root-dipped into rhizobacterial antagonist suspension containing 10^8cfu/mL and sterile distilled water for control. Foliar sprays of each rhizobacterial antagonist were applied 28 and 35 days after transplanting (DAT). Inoculation of bacterial leaf blight (BLB) pathogen was done at 40 and 45 DAT using leaf cutting method (Gnanamanickam, Velusamy, & Immanuel, 2013, pp.356-362). Percent of disease suppression was calculated by measuring the mean lesion length of the inoculated control plants minus the mean lesion length of the rhizobacterial antagonist treated plants over the mean lesion length of the inoculated control plants multiply by 100. There were 20 random plants with three replications in these experiments. Table 1 shows that the disease severity was evaluated 18 days after inoculation (DAI) up to 56 DAI with seven days interval following the modified rating scale of International Rice Research Institute –Standard Evaluation System for BLB percent infection following the formula:

$$\% \text{ Disease Severity} = \frac{0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5 + \ldots\ldots + 9n_9}{9N} x100$$

Where:

n = number of samples showing the scale of 0, 1, 2, 3 and 4, 5 to 9, respec tively

N = total number of samples

9 = represents the highest scale

Statistical Analysis

Both in vitro and in vivo screening of the potential rhizobacterial antagonists were conducted using Complete Randomized Design (CRD) with three replications. Statistical analyses were carried out using Stata/IC 12.1. Experimental data were subjected to analysis of variance (ANOVA) in which mean values of the different variables were compared, and statistical interactions were investigated. To determine which means were significantly different ($p < 0.01$), multiple comparisons tests Duncan's new multiple range test (DMRT) were used.

Table 1

Modified Disease Severity Rating Scale Used to Assess Symptoms caused by Bacterial Leaf Blight (Xanthomonas oryzae pv. oryzae) in Rice Plants in Greenhouse Test (IRRI- SES 1996)

Index	Percent Leaf Infection
0	0%
1	1-3%
2	4-6%
3	7-12%
4	13-25%
5	26-50%
6	51-75%
7	76-87%
8	88-94%
9	95-100%

RESULTS AND DISCUSSION

***In vitro* Screening of the Potential Rhizobacteria**

Table 2 illustrates that in the current study, seven out of twenty-three rhizobacterial isolates inhibited the growth of Xoo. However, only three showed a strong degree of antagonism exhibiting a wide, pronounce zone of inhibition. The three isolates, namely: FVP 08 (31. 23 mm), FVP 09 (41 mm), and FVP 22 (54.7 mm) continued to inhibit the growth of Xoo at 72 hours incubation. The inhibition of growth could have been due to the metabolites produced by these isolates. Beric and co-workers (2012), found out that large a number of rhizobacteria produced bacteriocin, contained genes involved in the biosynthesis of lipopeptides of the iturin and surfactin classes, carry the sfp gene, responsible for the biosynthesis of surfactin with potential for biological control against Xoo.

***In vivo* Screening of the Rhizobacterial Antagonists.**

The mean BLB lesion length in the rhizobacterial antagonists-treated plants shows significant difference. Table 3 reveal that among the isolates, FVP 22 treated plants showed the short-

est mean lesions with only 22.32 cm followed by FVP 09 and FVP 08 treated plants with 27.25 cm and 31.63 cm respectively, compared to the control plants with 46.97 cm.

Table 2

Zone of Inhibition (mm) of the Rhizobacterial Antagonists against Xanthomonas oryzae pv. oryzae at 24, 48 and 72 hours after incubation

Rhizobacteria Isolates	Hours of Incubation		
	24	48	72
FVP08	14.43[ab]	25.27bc	31.23b
FVP09	19.33[ab]	33.67c	41.00c
FVP11	13.33[ab]	14.03ab	14.03a
FVP14	11.23[ab]	11.43a	11.43a
FVP15	14.70[ab]	14.87ab	14.87a
FVP17	11.77[ab]	11.83a	11.83a
FVP22	21.27[ab]	49.10d	54.70d
F-test	**	**	**
C.V.	4.63	3.88	2.53

**Duncan's multiple range test (P<.0.01)

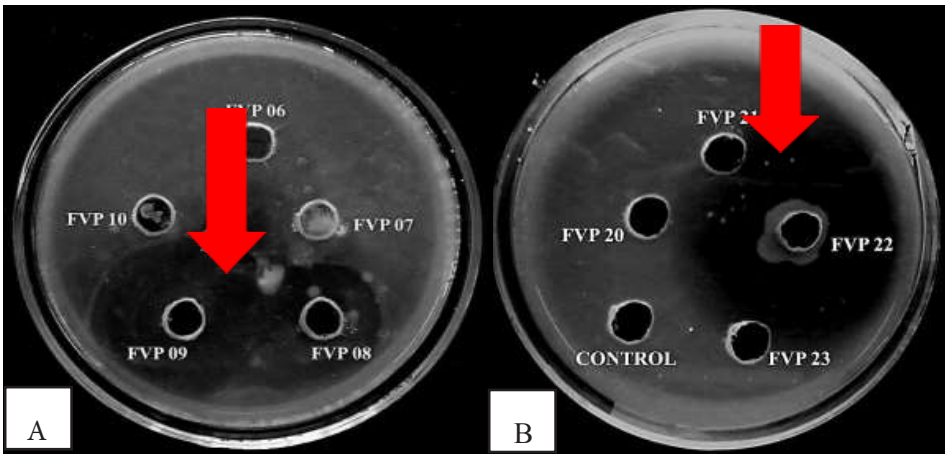

Figure 2. *In vitro* test for antagonism of rhizobacterial isolates (a) FVP 08 and FVP 09 and (b) FVP 22 against *Xanthomonas oryzae* pv. *oryzae* at 72 hours

The control plants showed severe BLB disease symptoms with pronounced, long and spreading blight lesions extending to the near base of the infected leaves, while the plants treated with the rhizobacterial antagonists have relatively shorter lesions. Table 3 further shows that the three isolates significantly suppressed BLB lesions with FVP 22 by 52. 48% followed by FVP 09 and FVP 08 with 42.30% and 32.36% respectively. These findings conform with the relative results from Gnanamicknam et al., (2013) on the different rhizobacteria isolated in rice ecosystem that suppressed the rice BLB by 58.83% and 51.88% under glasshouse and field conditions, respectively. Thus, different species of these rhizobacteria are applied to rice plants as a seed treatment before sowing, root dip prior to transplanting and two foliar sprays prior to inoculation can suppress *Xoo* by up to 59% (Islam, Pamplona, Atkinson, & Azucena, 2013). Moreover, figure 3 illustrates that the rhizobacterial antagonists significantly reduced the percent

BLB severity. Table 3 and figure 4 both show that from 18 to 56 DAI, plants treated with FVP 22 got the lowest BLB severity with 42.59% followed by FVP 09 and FVP 08 with 44.44% and 48.89% respectively compared to the control plants with of 60.47% BLB severity. Antagonistic potential of different rhizobacteria were studied by several workers including Gnanamanickam (2013) and Gangwar (2013) found that bacterization of rice with these rhizobacteria

Table 3

Effects of Rhizobacterial Antagonists on the Lesion Length, Disease suppression and severity on inoculated Rice Plants at 56 days after Inoculation

Treatments	Lesion Length (cm)	Percent Disease Suppression	Percent Disease Severity
FVP08	31.63c	32.36a	48.89a
FVP09	27.25b	42.30b	44.44a
FVP22	22.32a	52.48c	42.59a
CONTROL	46.97d	_ _	60.74a
F - test	**	**	**
CV	0.38	0.99	0.82

**Duncan's multiple range test (P<0.01)

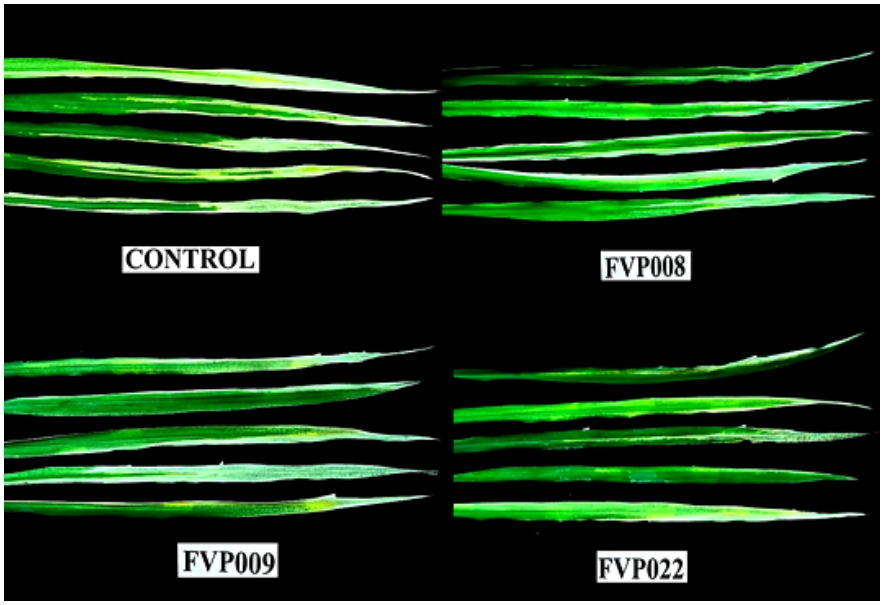

Figure 3. Suppression of bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) lesion lengths as affected by the different rhizobacterial antagonists

were studied by several workers including Gnanamanickam (2013) and Gangwar (2013) found that bacterization of rice with these rhizobacteria followed by its foliar sprays caused 40 to 60% reduction in bacterial leaf blight. The possibilities of Induced Systemic Resistance (ISR) were also considered for the management of the bacterial leaf blight in this study. Kloepper, Ryu, & Zhang (2007) noted in their review on the ISR of some rhizopheric bacteria like *Bacillus spp*. and *Serratia spp*. that the protection resulting from elicitation has been reported against major plant pathogens. Several specific strains of species *B. amyloliquifaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B.mycoides, and B. sphaericus.* In most cases, *Bacillus spp*. that elicits ISR also elicits plant growth promotion. Studies on mechanisms indicate that elicita-

tation is asssociated with ultrastructural changes in plants during pathogen attack and with cytochemical alterations. Investigation into the signal transduction pathways of elicited plants suggest that Bacillus spp. activate some additional pathways (Choudhary et al., 2007).

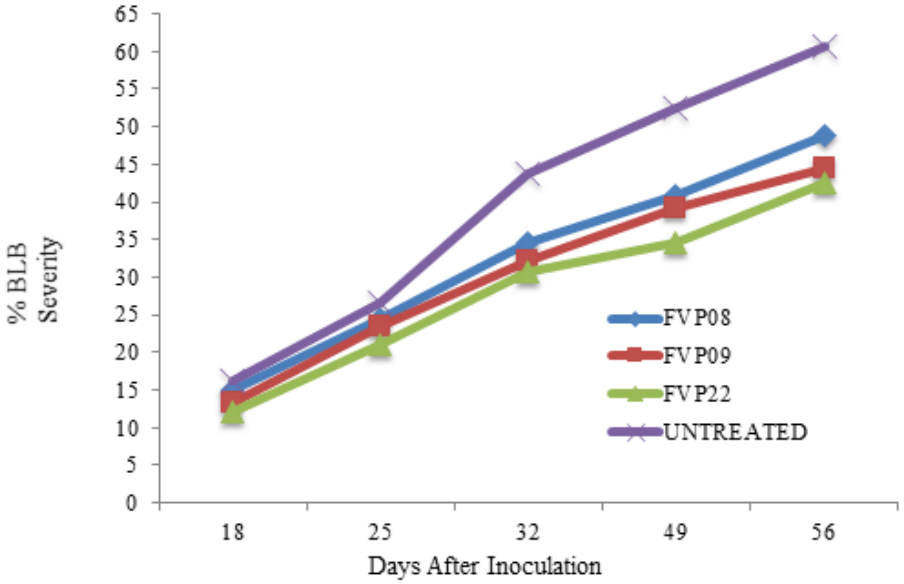

Figure 4. Percent bacterial leaf blight severity as affected by different rhizobacterial antagonists at 18 to 56 days after inoculation

CONCLUSION

It can, therefore, be concluded that the three antagonist rhizobacterial isolates effectively inhibit the growth of the bacterial leaf blight pathogen (*X. oryzae pv. oryzae*) and suppressed bacterial leaf blight severity when applied as seed soaking, root dipping prior to transplanting, and foliar sprays at 40 and 45 days after transplanting.

RECOMMENDATIONS

The *in vitro* and *in vivo* screening of the rhizobacterial isolates showed great success in inhibiting and suppressing the bacterial leaf blight pathogen. Thus, the success both in these two sets of conditions suggests further evaluation and tests. The morphological and biochemical tests should be undertaken to confirm the identities of the three local rhizobacterial antagonists. Further studies on the isolation and characterization of the possible antibiotic volatiles/secondary metabolites produced by these antagonist rhizobacterial isolates capable of inhibiting the growth of the bacterial leaf blight pathogen may be conducted. Further tests and investigation on the possible growth-promoting hormones produced by the antagonist rhizobacterial isolates associated with rice may be done.

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