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Application of *Zea mays* L. Rhizospheric Bacteria as Promising Biocontrol Solution for Rice Sheath Blight

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ABSTRACT

Sheath blight is referred to be a serious soil-borne disease resulting in financial losses towards rice farming. The existing research focused towards examining the potential of *Bacillus subtilis* strain AK38 (GenBank ID: KY458554. 1) and *Pseudomonas fluorescens* strain AK18 (GenBank ID: KY458552. 1), isolated from maize (*Zea mays* L.) rhizosphere, to regulate sheath blight caused by *Rhizoctonia solani* in rice (*Oryza sativa* L.) as well as to examine their impact on plant development. Biocontrol attributes of selected strains, biofilm examination, root colonisation and gnotobiotic examination had been determined. AK38 and AK18 bacterial strains created biofilm effectively and live in rice rhizosphere even after 30 days of the plantation with 5.2×10^5 and 4.8×10^5 CFU/g of root. The quantity of auxin synthesis was registered 31.2µg ml^{-1} in the 72 hr of incubation. Additional plant development attributes i.e. siderophore production, phosphate solubilization, HCN production was confirmed positive with regard to each isolate. The statistical study of data shown significant improvement in root and shoot size 95% and 78.4%, respectively, over control. In addition, 77% decline within disease incidence has been demonstrated *in vivo* trials.

Keywords: Biocontrol, biofilm, rhizosphere, PGP, sheath blight, *Zea mays* L.

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INTRODUCTION

At ongoing annual rate, the entire world population will be expected to expand at 1.2% or even approximately seventy seven million individuals per year**.** As documented by United Nations, the global human

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population is anticipated to rise through 7.6 billion within 2017 to 8.6 billion within 2030, 9.8 billion in 2050 and 11.2 billion in 2100 (Van Bavel, 2013). According to the results of the United Nations 2017 Revision (Food and Agriculture Organization [FAO], 2017), the world's population numbered nearly 7.6 billion as of mid-2017, implying that the world has added approximately one billion inhabitants over the last twelve years. Sixty per cent of the world's people live in Asia (4.5 billion), 17 per cent in Africa (1.3 billion), 10 per cent in Europe (742 million), 9 per cent in Latin America and the Caribbean (646 million), and the remaining 6 per cent in Northern America (361 million) and Oceania (41 million). China (1.4 billion) and India (1.3 billion) remain the two most populous countries of the world, comprising 19 and 18 per cent of the global total, respectively. Irrespective of significant innovations within farming technology over the previous fifty years, substantial volumes of the world's population are still affected from starvation (Table 1) and undernourishment (FAO, 2009). With this particular population increase, it is anticipated that an identical food security challenge may arise with the chances associated with losing farming area due to industrialization and urbanization. Along with existing cultivated fields complications, existing and new plant diseases raise the difficulties for farmers and make it challenging to meet up with the global nutritional requirement for increasing population (Satterthwaite, McGranahan, & Tacoli, 2010).

Rice is an important cereal right after wheat and maize, on which human society largely relies for their nutritional demands (Nadeem et al., 2016). Rice delivers 27 % carbohydrate utilized as dietary energy supply and twenty percent associated with dietary proteins consumption (Muthayya, Sugimoto, Montgomery, & Maberly, 2014). Rice is cultivated through a number of regions and weather conditions. India, China, Pakistan, and Bangladesh are primary producers (Table 2) and consumers associated with rice food. Within India, rice is cultivated under varied environments like rainfed uplands, rainfed shallow, semideep and deepwater lowlands, irrigated lands and hillsides. No other plant varieties are able to cultivate under this kind of broad selection of environmental conditions. On a yearly basis, 148 million hectares (m ha) are sown to rice globally, including 79 m ha (53%) in irrigated environment, 17 m ha (12%) within rainfed uplands 41 m ha (27%) in rainfed lowlands and 11 m ha (8%) in flood prone environment (Haefele, Nelson, & Hijmans, 2014; Singh, McClean, Büker, Hartley, & Hill, 2017).

Sheath blight (ShB) of rice induced by *Rhizoctonia solani* Kuhn (Teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is known as a key biotic concern associated with rice in the majority of the rice cultivating nations of Asian countries (Jisha & Shabanamol, 2014; Zhao et al., 2016). *Rhizoctonia solani* is polyphagous competitive saprophyte and has a broad host selection. Crop deficits usually differ from 0 to 50% based on intensity of the infection

Table 1

Prevalence of undernourishment in the world by region, 2000–2016 (FAO, 2017)

	2000	2005	2010	2011	2012	2013	2014	2015	2016
	Percentage								
WORLD	**14.7**	**14.2**	**11.5**	**11.2**	**11.0**	**10.8**	**10.7**	**10.6**	**11.0**
AFRICA	**24.3**	**20.8**	**18.3**	**17.9**	**17.8**	**17.8**	**18.1**	**18.5**	**20.0**
Northern Africa	**6.8**	**6.3**	**5.1**	**4.8**	**8.5**	**8.4**	**8.3**	**8.3**	**8.3**
Sub-Saharan Africa	**28.1**	**23.7**	**20.6**	**20.2**	**20.0**	**20.0**	**20.4**	**20.8**	**22.7**
Eastern Africa	39.3	34.3	30.9	30.2	30.6	30.6	30.9	31.1	33.9
Middle Africa	37.4	29.4	23.8	23.1	22.5	22.3	24.0	24.4	25.8
Southern Africa	7.1	6.4	6.7	6.3	6.2	6.2	6.5	6.6	8.0
Western Africa	15.1	12.0	10.0	9.9	9.9	9.8	9.8	10.4	11.5
ASIA	**16.7**	**17.0**	**13.2**	**12.8**	**12.5**	**12.2**	**11.9**	**11.6**	**11.7**
Central Asia and Southern Asia	**17.6**	**20.1**	**15.7**	**15.7**	**15.6**	**15.4**	**15.1**	**14.7**	**14.2**
Central Asia	15.7	14.2	10.6	9.9	9.1	8.4	8.2	8.2	8.4
Southern Asia	17.7	20.4	15.9	15.9	15.9	15.7	15.3	14.9	14.4
Eastern Asia and South-Eastern Asia	**16.6**	**15.2**	**11.6**	**10.9**	**10.4**	**9.9**	**9.6**	**9.2**	**9.7**
Eastern Asia	14.6	14.1	11.3	10.7	10.3	9.9	9.5	9.1	9.0
South-Eastern Asia	22.0	18.1	12.4	11.3	10.7	10.0	9.7	9.4	11.5
Western Asia	**11.3**	**10.5**	**9.4**	**9.1**	**8.9**	**8.7**	**8.9**	**9.3**	**10.6**

Table 1 *(Continue)*

LATIN AMERICA AND THE CARIBBEAN	**12.0**	**9.1**	**6.8**	**6.6**	**6.4**	**6.3**	**6.3**	**6.3**	**6.6**
Latin America	**11.1**	**8.0**	**5.9**	**5.7**	**5.5**	**5.4**	**5.4**	**5.5**	**5.9**
Central America	8.1	8.3	7.1	7.2	7.1	7.1	6.9	6.7	6.5
South America	12.2	7.9	5.4	5.1	4.8	4.7	4.8	5.0	5.6
Caribbean	**23.8**	**23.3**	**19.9**	**19.3**	**19.4**	**19.2**	**18.9**	**18.4**	**17.7**
OCEANIA	**5.3**	**5.3**	**5.0**	**5.2**	**5.3**	**5.7**	**6.0**	**6.4**	**6.8**
NORTHERN AMERICA AND EUROPE	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**	**< 2.5**
Other country group: Western Asia and Northern Africa	9.3	8.7	7.6	7.3	8.7	8.5	8.6	8.8	9.5

Table 2

Top 5 Rice Producing Countries (FAO, 2018)

Rank	Country	Rice Production (metric tonnes)	% of World Total
1	China	206,507,400	27.8%
2	India	157,200,000	21.2%
3	Indonesia	70,846,465	9.5%
4	Bangladesh	52,325,620	7.0%
5	Vietnam	44,974,206	6.0%

as well as the development cycle at which the crop is attacked and environmental situations (Silva, Figueiredo, Andreote, & Cardoso, 2013; Toan et al., 1997). Preliminary indications of sheath blight come in the form associated with spherical, rectangular or ellipsoid, greenish, greyish, water-soaked areas of about 1 cm long that appear on leaf sheaths close to the water line (Toan et al., 1997). The disease develops quickly during flowering when the rice canopy is most dense, forming a microclimate favourable to pathogen growth and spread (Silva et al., 2013). *R. solani* can infect seed to fully mature plant, causing moderate to significant yield losses depending on the plant part affected. Visible plant disease symptoms include formation of lesions, plant lodging, and presence of empty grains. Large lesions formed on infected sheaths of lower rice leaves may lead to softness of the stem thereby initiating stem lodging (Wu et al., 2012). Lodging alters the normal rice canopy design, affecting photosynthetic ability and total biomass production (Silva et al., 2013).

The current research aimed at analyzing the potential of *Bacillus subtilis* strain AK38 (GenBank ID: KY458554. 1) and *Pseudomonas fluorescens* strain AK18 (GenBank ID: KY458552. 1) species to control sheath blight in rice. Various plant development attributes associated with bacterial strains i.e. siderophore production, HCN production, IAA production were also determined.

MATERIALS AND METHODS

Strains

Bacterial isolates intended for current research were isolated and identified from *Zea mays* L. rhizosphere as explained in earlier investigation of Karnwal (2017). *Bacillus subtilis* AK38 (GenBank ID: KY458554. 1) and *Pseudomonas fluorescens* AK18 (GenBank ID: KY458552. 1) were preserved on nutrient agar medium (NAM) at 4°C. Fungal strain, *Rhizoctonia solani* Kuhn, was procured from Indian Agricultural Research Institute (IARI, India) and grown on potato dextrose agar (PDA). The biocontrol potential of bacterial isolates was determined by implementation of dual-culture technique.

Dual Culture

Bacterial isolates were grown in nutrient broth at 150 rpm (3 x *g*) for 24h at 30°C in rotatry shaker incubator. After incubation, bacterial cultures were centrifuged at 6000 rpm (4025 x *g*) for 10min at 4°C. The broth was decanted and bacterial pellets were re-suspended in sterile distilled water. Bacterial cells were counted using a viable plate count and optical density methods on NAM plates, and adjusted to a concentration of 10^8 colony forming unit (CFU) mL^{-1} (OD = 0.5) at 600 nm.

The dual culture / antibiosis assay was performed on PDA in 90 mm diameter petri plates (Khaledi & Taheri, 2016). Fungal pathogen disk of 5mm was placed in the centre of PDA plates whereas 10µL of the bacterial suspensions were uniformly

distributed around the fungal disk at a distance of 20 mm. Dual culture plates were incubated at 28 °C for 48 h. Each combination was replicated 10 times. As negative controls, 5 Petri dishes with PDA were inoculated only with an *R. solani* and 10µL sterilized distilled water at a distance of 20 mm from fungal pathogen.

Bio-film Assay

For biofilm assay AK18 and AK38 bacterial strains were cultured in Luria Bertani (LB) medium and incubated at 37°C for 24 h. Incubated bacterial culture were transferred in four-well polystyrene plates containing casein digest-mannitol medium (Heidarzadeh & Baghaee-Ravari, 2015). These bacteria inoculated polystyrene plates were incubated for 3 days without shaking at 37°C. Three days after, polystyrene plates were rinsed with sterilized distilled water to remove the medium from wells and placed for drying at 37 °C for 30 min. Immediate after drying wells were stained with 1% w/v crystal violet and biofilm development was determined by calculating the OD500 per well using a plate reader. The complete procedure for biofilm formation was repeated three times to reduce the error during experiment.

Biocontrol (Chitinase Assay) and Growth Traits of Bacterial Strains

To access the possibilities of both bacterial isolates as promising biocontrol agent towards fungal pathogen, a substrate depending approach was applied. Bacterial cultures were inoculated into modified LB plate consisting 0. 2% colloidal chitin and 1.5% agar. Modified LB plates were cultivated at 35 °C for 72 h in order to visualize the hollow region of chitin hydrolysis by chitinase enzyme released by bacterial isolates. In addition, plant development traits like auxin formation, siderophore formation, and phosphate solubilization were examined for every single bacterial strain as described by Karnwal (2017).

Seedling Incubation and Inoculation

Selected bacterial strains are potentially amoxicillin resistant (Karnwal, 2017), so for root colonization study each bacterial isolate were grown on amoxicillin amended nutrient broth with 200 µg ml^{-1} of amoxicillin concentration. Rice seedlings were dipped in bacteria inoculated nutrient broth having 10^8 CFU ml^{-1} bacterial cells and incubated in plant growth chamber. After 30 days, bacteria inoculated rice roots were collected and 1 g of root was gently crushed in normal saline and 100 µl serially diluted sample was spreaded on NAM plated having 200 µg ml^{-1} amoxicillin. These plates were incubated at 32°C for 48 h and colony forming unit (CFU) per g of root was calculated as described by Heidarzadeh and Baghaee-Ravari (2015).

In vivo* Antagonism of Tested Bacterial Isolates against *R. solani

To identify the biocontrol potential of bacterial isolates towards experimental phyto-pathogenic fungi *in vivo*, rice

seeds were exposed with bacterial inoculum priory, before exposure to the phyto-pathogenic fungi. Rice seeds were pre-germinated in dark on clean and sterile moist cotton bed in Petri dishes under laminar hood for five days. These seeds were sprayed with bacterial culture having 10^8 cells ml^{-1} of bacterial cell concentration and then germinated in water agar plates for 2 days. After germination, seedlings were shown and incubated along with seven days older fungal culture at 25°C in dark for 2 days. Four replicates of each treatment were carried out to get suitable data regarding statistical evaluation. In control treatment, seeds were exposed to *R. solani* Kuhn and germinated upon water agar plates alone.

Greenhouse Study

Green house study was performed with 14 days old bacterial treated and fungal pathogen contaminated rice seedlings. Seedlings with fungal culture (1:1) were planted in sterilized earthen pots having sterilized sandy loam soil. For increasing bacterial population around root of seedlings, fresh bacterial culture was inoculated around the roots without damaging rice roots. In control (with fungal pathogen, without bacteria treatment) sterilized water was poured for comparison between bacterial treated and non treated trials. These planted pots were kept for 30 days in Greenhouse to report the sheath blight incidence. To achieve the appropriate outcomes, comparison was carried out after 10 and 20 days by using five disease scales mentioned by Chen, Bauske, Musson, Rodriguezkabana and Kloepper (1995): 0 = no disease; 1 = 0-25% of the leaves withered; 2 = 26-50% of the leaves withered; 3 = 61-75% of the leaves withered; 4 = 76-100% of the leaves withered. Disease index was calculated by applying following formula (Heidarzadeh & Baghaee-Ravari, 2015):

$$\Sigma [(P \times DC) \times 100]/(T \times 4),$$

where P = plants per class, DC = disease index and T = total number of plants.

Percent efficacy of disease control was also measured as described by Purkayastha, Saha and Saha (2010)

[(DC of control - DC of bacterial inoculated plants)/DC of control] × 100

Statistical Analysis

Statistical data analysis was performed by using SPSS 16 software for experimental data. To analyze the vital differences among treatments, Fisher's protected LSD was applied with 5% probability level by using Statistical Analysis System software (Karnwal, 2017).

RESULTS

The results of the dual culture study demonstrated that both *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 had antagonistic effect on *Rhizoctonia solani* Kuhn. The results produced by antagonists were significantly ($P < 0.05$) different from control as well as within them. *Bacillus subtilis* strain AK38 highly inhibited the growth of test pathogen compared to *Pseudomonas*

fluorescens strain AK18, and the percentage of inhibition increased about two times to AK38 and three times to AK18 from 48 hours to 72 hours incubation (Table 3).

Table 3

Effect of AK38 and AK18 bacterial isolate on the radial growth of R. solani *in dual culture method*

Antagonists	Radial growth at 48 hours*		% inhibition	Radial growth at 72 hours*		% inhibition
	Control	Test		Control	Test	
AK38	64.3 mm	45 mm	30.0 %	72 mm	20.8 mm	71.1 %
AK18		49.2 mm	23.5 %		24 mm	66.7 %

* Values are mean of three replicates

Colonisation Potency of Bacterial Isolates

Preliminary experiments confirmed the antifungal activity of *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 against *Rhizoctonia solani* Kuhn. Chitinase assay results reported as a clear zone of chitinase activity around bacterial growth. In addition to biocontrol activity, AK38 and AK18 were capable to form biofilm in polystyrene plates. Current study results revealed that AK38 to be more efficient for biofilm formation over AK18 isolate. Colonization studies demonstrated that AK38 and AK18 strains could colonise and live successfully in rice rhizosphere with the density of 5.2×10^5 and 4.8×10^5 CFU/g of root, respectively, after 30 days of treatment.

Phyto-stimulatory Effect of Bacterial Isolates

Both isolates produced detectable IAA concentrations in medium with L-tryptophan (Table 4). At 50 µg ml^{-1} of L-tryptophan AK38, and AK18 released significant concentrations of indole (2.6 µg ml^{-1} and 1.4 µg ml^{-1}, respectively) in contrast to 0 µg ml^{-1} of L-tryptophan concentration. Significant amount of IAA was detected with 100 µg ml^{-1} tryptophan produced by isolates AK38 and AK18 (6.0 µg ml^{-1} and 4.0 µg ml^{-1}, respectively). A considerably higher concentration of IAA synthesis by AK38 and AK18 was noted when 500 µg ml^{-1} L-tryptophan was supplied to the isolates (Table 4).

Accessibility to iron within environment act as a vital limiter for development of living cells, microorganisms, plants, and animals. Many workers (Khaledi & Taheri, 2016; Nadeem et al., 2016) observed that bacterial siderophores could become an effective source to fulfill the need of soluble iron for the host plant and helped in plant growth. In the present study, AK38 and AK18 strains produced orange clear zone around the bacterial growth on CAS agar (Table 4). Bacterial isolate AK38 and AK18 also developed translucent clear zone around the bacterial growth on Pikovskaya's agar plates and confirmed liquefaction of inorganic phosphate by bacteria.

Effectiveness of Antagonists in The Pot Trials in Growth Chamber

In vivo study with two biocontrol agents in rice plant resulted significant decline in various development constraints of rice over controls. Experimental data was statistically analysed through ANOVA by using mean values of four replicates in which treatments

Table 4
Characterisation of IAA and biocontrol traits in antagonistic bacterial isolates

Isolate	L-tryptophan concentration for IAA production (µg ml^{-1})				Biofilm formation	Siderophore production	Chitinase production
	0	50	100	500			
AK38	0.2	2.6	6.0	10.0	+	+	+
AK18	0.2	1.4	4.0	9.9	+	+	+

+: Positive; −: Negative

Table 5

Rice Sheath blight disease control by bacterial isolates

	After 10 day inoculation		After 20 days inoculation	
	Disease index	% efficacy of disease control	**Disease index**	**% efficacy of disease control**
AK38	20.1 ± 1.3	70 ± 1.3	18.2 ± 1.3	76 ± 1.1
AK18	31.2 ± 0.7	55 ± 0.7	29.8 ± 0.9	61 ± 0.5
Control	67.1 ± 1.2	0.0 ± 0.0	73.7 ± 1.6	0.0 ± 0.0

Table 6
Plant growth promotion effect of bacterial isolates under green house condition on rice

Strain	Shoot length (cm)	Root length (cm)	Shoot dry weight. (mg)	Root dry weight. (mg)
AK18	**21.2ab**	**4.1a**	**503.8a**	**76.3a**
AK38	**22.3a**	**3.8b**	**401.0b**	**65.3b**
Zero Control	**12.5c**	**2.1c**	**156.4c**	**17.7c**
LSD value	**1.17**	**0.63**	4.86	**2.64**

Means sharing the same letter(s) within a column did not differ significantly ($P \leq 0.05$)

disease index and disease control efficacy (Table 5). The antagonistic bacteria induced much less greenish grey spots on sheaths with low disease intensity and improved were examined using least significant differences ($p \leq 0.05$). Under greenhouse research, AK38 and AK18 isolates induced shoot growth, root growth and dry weight

significantly (Table 6). Statistical analysis revealed significant increment in root and shoot length with 80.9 and 95% increase in root and 78.4 and 69.6% raise in shoot length with AK38 and AK18, respectively (Table 6). Greenhouse study results proved the beneficial effect of both isolates through a significant increment in shoot fresh weight with AK38 and AK18 against uninoculated controls in presence of fungal pathogen.

DISCUSSION

It was detected by researchers (Ignatova, Brazhnikova, Berzhanova, & Mukasheva, 2015) that varied concentrations of L-tryptophan perform a significant function in determining the concentration of IAA synthesis by microorganisms under trials. Results of the present study supports the earlier published reports (Palacios, Gomez-Anduro, Bashan, & de-Bashan, 2016) regarding the effect of varied L-tryptophan concentrations regulating the biosynthesis of IAA and plant development (Chaiharn & Lumyong, 2011; Karnwal, 2009; Karnwal, 2017). Presence of iron in soil or on root surface encourage competition among soil micro-organisms (Sadeghi et al., 2012). Raupach and Kloepper (1998) reported the impact of iron chelater's (siderophores) produced by rhizospheric bacteria on plant development by increasing the bioavailability of soluble iron in the rhizosphere region. Phosphorus is a macronutrient that is required by all of living organisms. However, plants required this particular macronutrient in an extremely lesser volume although a critically low availability could lead to deficiencies and adverse impact on plant growth (Yasmin, Rahman Bakar, Malik, & Hafeez, 2004). Within soil maximum quantity of phosphorus is existing in solid or powder form that could not be directly utilized by plant. Research workers have documented the usage of soil residing bacteria for liquefaction of mineral phosphates into a plant utilizable form. Soil bacteria synthesized different organic acids for phosphate liquefaction. These types of organic acids ensure the bioavailability of insoluble mineral phosphate into soluble phosphate by acidification process (Zhang et al., 2015). Greenhouse study results are in conformity with other workers study (Balseiro-Romero et al., 2017; Kuan, Othman, Abdul Rahim, & Shamsuddin, 2016) those documented the beneficial effect of indole acetic acid secreted by *Bacillus subtilis*, which often favours plant development by maximizing the amount of root hairs. In order to provide an advantageous impact by PGPR, the colonization associated with bacteria within the plant rhizospheric zone is the most important aspect (Kuan et al., 2016; Zhang et al., 2015). However various other factors i.e. phytohormone formation, eradication of pathogenic microorganisms, phosphate solubilisation, and favouring the inorganic nutrient uptake are also considered to be associated with plant development supported by PGPR (Palacios et al., 2016; Sallam, Riad, Mohamed, & El-Eslam, 2013).

CONCLUSION

This study demonstrates that *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 isolated from maize rhizosphere were capable of suppressing the growth of *R. solani in vitro*. The inhibition zones produced by the test isolates of antagonistic bacteria greatly varied. From this result, it could be said that biological control might be effective and alternative in minimizing the incidence of the disease. A significant percent control of sheath blight was observed when seeds soaked with the test bacteria. Both isolates have a great potential as a promising biocontrol agent and offers a good prospect for integrated management of the sheath blight of rice. As well as colonization and phyto-stimulatory study reveal the positive aspect of isolates as promising biofertilizer agents. However, additional research on *Bacillus subtilis* strain AK38 and *Pseudomonas fluorescens* strain AK18 is still needed to proceed further with selected BCA addressing sheath blight disease control under rainfed lowland culture as well as in several areas including i.e. its formulation and applications; repetition of *in vivo* studies with other crops and integration into a production system.

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