Indian J. Applied & Pure Bio. Vol. 39(1), 486-495 (2024). **ISSN: 0970-2091**
A Web of Science Journal

Isolation, morphology characterization and an Antagonistic activity of *Bacillus subtilis* against Sheath Blight of Rice (*Oryza sativa* L.) caused by *Rhizoctonia solani* Kuhn.

R. Kannan and K. Sanjeevkumar

Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Annamalai Nagar- 608 002 (India)
email: pathosanjeev@yahoo.co.in

Abstract

Bacillus subtilis is one of the major plant growth promoting rhizobacteria (PGPR) and act as predominant bio control agents against many plant pathogens of crop plants. The present study was made to isolated the different *Bacillus* strains and selected to assess their capability to inhibition of rice sheath blight pathogen. Among the different isolates *Bacillus* sp. tested, based on the topography, shape, colour and colony characters of *Bacillus* sp. the isolates collected were grouped into five categories. All isolates were proved to be Gram positive. Positive results were obtained for Starch hydrolysis, Gelatin liquefaction Casein Hydrolysis, IAA production, HCN Production and Catalase test. Similarly, negative reaction was obtained for pigement test, Methyl red test, H2S test and Urease utilization confirming the isolates to be *Bacillus* spp. The isolate *B. subtilis* (AUBS 2) was found to be the most effective against *R. solani* in dual culture. In poisoned food technique, the mycelial growth and sclerotial germination of *R. solani* was found to be reduced with an increase in the conc. of culture filtrate of *B. subtilis*. The culture filtrate of *B. subtilis* at 20 per cent conc. completely inhibited the mycelial growth and sclerotial germination of *R. solani*.

Key words : *Bacillus subtilis, R. solani,* Isolation, morphology characterization.

The antagonistic species of Bacillus act as a biocontrol agent has been studied. Their potential of biocontrol agents is investigated because they produce kinds of antimicrobial compounds, including peptide and hydrolytic enzymes. Antagonistic microbes to minimize the use of chemical pesticides has recently become more prevalent. In an attempt to find out the isolation and morphological characterization of *Bacillus* at different localities and antagonist activity for sheath blight caused by *Rhizoctonia solani* Kuhn. in rice. (Teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk) anastomosis group 1 and subgroup 1A). Rice sheath blight (caused by *R. solani*) is a soil borne disease of rice, occurs throughout

temperate and tropical production areas and is most prominent where ever rice is grown under intense production systems[20] and is second only to rice blast as the most economically important fungal disease of rice[19]. Biological control of plant pathogens and its utilization in rice ecosystem though gaining popularity in majority of crops is still at its infancy due to its varied reasons. The survival growth and establishment of biological control agents in rice which is a crop that is grown under submerged conditions, is questionable. However, effective management strategy of sheath blight disease is feasible only when biocontrol agents in rice based cropping system survive, establish, proliferate and control sheath blight pathogen and also have a synergistic growth promoting effect on the crop. Under such circumstances use of Plant Growth Promoting Rhizobacteria (PGPR) offer a promising means of controlling diseases and improve the yield in the rice ecosystem[11].

Several chemicals have been suggested to control rice diseases but the recommended fungicides such as Zineb, Dithane M 45, carbendazim, validamycin, propiconazole and mancozeb and *etc*., do not provide satisfactory disease control under heavy inoculum pressure[7]. Also, the possibilities of the pathogen developing resistance against the pathogen necessitate the search for new molecules of fungicides. However, it is imperative that with the growing concerns on environmental pollutions due to chemical pesticides, there is a felt need to reduce the amount of chemical being used and also to reduce the number of applications. Towards this direction the present study was undertaken with the following objectives. Isolation, morphology characterization and an Antagonistic activity of *Bacillus subtilis* against Sheath blight of rice.

Isolation of B. subtilis isolates from phylloplane of rice crop :

Healthy leaves were collected from paddy fields in different rice growing areas of Tamil Nadu at grain formation stage. For isolation of bacteria two grams of each sample were washed with sterile water and blot dried. The samples were cut into small pieces of 5mm size and taken into 250 ml Erlenmeyer flask with 20 ml of sterile distilled water (SDW). After shaking with an orbital shaker at 150 rpm at 28±2°C for 24 hrs, serial dilutions (10^{-1} to 10^{-8}) of the suspension was made. Small aliquots (50 µl) from dilutes of 10^{-7} and 10^{-8} were lawned onto nutrient agar (NA) media in Petri dish. The plates were incubated at 28±2°C for 24-48 hr for colony formation. Selection of single bacterial colonies was done based on morphological variation and after purification they were preserved in refrigerator.

Morphological characterization of Bacillus isolates :

Fourteen bacterial isolates visually observed and identified based on colony shape, colony type and colour in accordance with the taxonomic literature.

Biochemical tests of Bacillus species :

Biochemical tests that investigate the enzymatic activities of cells are powerful tests as described by Ahmad *et al*.,[2] for the identification of *Bacillus* species. Bacterial isolates were examined for Gram reaction by

Ryu test[16]. The bacterial isolates was examined for Methyl red test[4], Gelatin liquefaction and Casein hydrolysis (Reynolds[14]), IAA Production test (Ahmad *et al*.[2]), HCN was determined as per Wei *et al*.,[24] and Urease activity[4]. The hydrolysis of starch was determined based on the ability of the isolates to hydrolyse starch (Eckford[5]). The amylase activity was screened by employing zone clearing technique, using starch agar medium[3]. According to Eckford[5], the acid and gas production of the bacterial isolates were also tested.

In vitro efficacy of biocontrol agents against R. solani :
Dual culture technique :

The antagonistic activity of bacterial bio control agents against *R. solani* was tested by dual culture technique. A 9 mm actively growing PDA culture disc of *R. solani* was placed at one end, 1.5 cm away from the edge. Just opposite to the pathogen one cm long streak of bacterial bio control agents was gently made in the medium using two days old culture at equidistance. A control was maintained by inoculating *R. solani* alone at one end of the Petri dish. The plates were incubated at room temperature (28±2°C) for seven days. Three replications were maintained for each antagonist. The radial growth (in mm) of the pathogen and the test antagonists and the extent of the inhibition zones (in mm) developed were measured. The effective antagonists were identified based on the inhibition of the growth of the pathogen. The radial mycelial growth of the pathogen and per cent reduction over control was calculated by using the formula proposed by Vincent[23].

$$\text{Per cent inhibition (I)} = \frac{C - T}{C} \times 100$$

Where, C-mycelial growth of pathogen in control,
T - Mycelial growth of pathogen in dual plate.

Among the antagonists, *B. subtilis* (AUBS2) recorded the maximum inhibition of the mycelial growth of *R. solani* and hence the same isolate alone was tested further using poisoned food technique (liquid medium assay).

Preparation of the culture filtrates of B. subtilis (AUBS2) :

B. subtilis (AUBS2) was inoculated into Erlenmeyer flasks containing 50 ml of sterile NA broth and kept on a rotary shaker at 100 rpm for 48 h. Then the culture was filtered through bacteriological filter under vacuum and the filtrate thus obtained was used for the subsequent studies.

Effect of culture filtrate of B. subtilis (AUBS2) on the mycelial growth of R. solani (Poisoned food technique) :

The culture filtrate of *B. subtilis* was separately incorporated into sterilized PDA medium at 5, 10, 15, 20 and 25 per cent by adding the calculated quantity of the culture filtrate to the medium by means of a sterile pipette. The PDA medium without the culture filtrate served as control. The amended media were transferred to sterile Petri dishes separately @15 ml and allowed to solidify. Each plate was inoculated at the centre with seven days old (9 mm) PDA culture disc of *R. solani*. Carbendazim 50% WP @ 0.1 per cent conc. was used for comparison. The diameter of the

Table-1. Morphological characteristics of *Bacillus* isolates

S. No.	Locality	Isolates	Cell shape	Colony type	Colour of the colony
1	Akkarai Jeyakondappattinam	AUBS 1	Rod	Circular, opaque, colony with serrated margin	Slightly yellow
2	Andarmullippallam	AUBS 2	Rod	Circular, opaque, colony with serrated margin	Creamy White
3	Ariyakoshty	AUBS 3	Rod	Circular colony with serrated margin	Creamy White
4	C. Manambadi	AUBS 4	Rod	Circular, opaque, colony with serrated margin	Creamy White
5	Karamedu	AUBS 5	Rod	Circular, opaque, colony with serrated margin	Creamy White
6	Killai	AUBS 6	Rod	Circular colony with serrated margin	Creamy White
7	Naduthittu	AUBS 7	Rod	Circular, opaque, colony with serrated margin	White
8	Pethanagkuppam	AUBS 8	Rod	Circular, opaque, colony with serrated margin	Slightly yellow
9	Ponnanthittu	AUBS 9	Rod	Circular, opaque, colony with serrated margin	Creamy White
10	Poochimedu	AUBS10	Rod	Circular, opaque, colony with serrated margin	Slightly yellow
11	Pudhupettai	AUBS 11	Rod	Round colony with smooth margin	White
12	Samiyar pettai	AUBS12	Rod	Circular, opaque, colony with serrated margin	Creamy White
13	Samuttykuppam	AUBS13	Rod	Circular, opaque, colony with serrated margin	Creamy White
14	Singarathoppu	AUBS 14	Rod	Circular, opaque, colony with serrated margin	Creamy White
15	T.S. Pettai	AUBS 15	Rod	Circular uniform colonies surrounded by zone of precipitation	Creamy White

mycelial growth (mm) of *R. solani* was measured when the mycelial growth fully covered the control plates.

Effect of culture filtrate of B. subtilis (AUBS2) on the mycelial dry weight of R. solani :

The culture filtrates of *B. subtilis* isolate were incorporated into sterilized PDA broth at 5, 10, 15, 20 and 25 per cent by adding the calculated quantity of the culture filtrate to the broth by means of a sterile pipette. The PDA broth without the culture filtrate served as control. Carbendazim 50 %WP @ 0.1 per cent conc. was used for comparison. The flasks were inoculated with nine mm mycelial disc of *R. solani* collected from the periphery

Table-2. Biochemical characteristics of *Bacillus* isolates

Sl. No.	Isolate	Gram staining	Pig-ment	Starch hydr-olysis	Gelatin liqui-faction	Methyl Red test	H_2S test	Casein Hydr-olysis	IAA produc-tion	HCN Produc-tion	Cata-lase test	Urease Utili-zation
1	AUBS 1	+	-	+	+	-	-	+	+	+	+	-
2	AUBS 2	+	-	+	+	-	-	+	+	+	+	-
3	AUBS 3	+	-	+	+	-	-	+	+	+	+	-
4	AUBS 4	+	-	+	+	-	-	+	+	+	+	-
5	AUBS 5	+	-	+	+	-	-	+	+	+	+	-
6	AUBS 6	+	-	+	+	-	-	+	+	+	+	-
7	AUBS 7	+	-	+	+	-	-	+	+	+	+	-
8	AUBS 8	+	-	+	+	-	-	+	+	+	+	-
9	AUBS 9	+	-	+	+	-	-	+	+	+	+	-
10	AUBS10	+	-	+	+	-	-	+	+	+	+	-
11	AUBS 11	+	-	+	+	-	-	+	+	+	+	-
12	AUBS12	+	-	+	+	-	-	+	+	+	+	-
13	AUBS13	+	-	+	+	-	-	+	+	+	+	-
14	AUBS 14	+	-	+	+	-	-	+	+	+	+	-
15	AUBS 15	+	-	+	+	-	-	+	+	+	+	-

Table-3. Effect of *Bacillus* isolates against *R. solani* (Dual Culture technique)

Sl. No.	Isolates	Mycelial growth of the pathogen (mm)	Per cent inhibition over control	Inhibition zone (mm)
1	AUBS 1	31.14	65.40	5.23
2	AUBS 2	20.12	77.64	9.35
3	AUBS 3	60.50	32.77	6.50
4	AUBS 4	78.31	12.98	2.70
5	AUBS 5	59.23	34.18	8.00
6	AUBS 6	34.51	61.65	1.50
7	AUBS 7	68.57	23.81	7.11
8	AUBS 8	43.58	51.57	2.21
9	AUBS 9	65.51	27.21	1.50
10	AUBS10	62.33	30.74	5.68
11	AUBS 11	65.52	27.20	5.13
12	AUBS12	28.22	68.64	6.54
13	AUBS13	25.56	71.60	9.00
14	AUBS 14	70.34	21.84	4.43
15	AUBS 15	27.33	69.63	8.67
16	Control	90.00	—	—
	S.Ed CD (p=0.05)		—	

Table-4. Effect of culture filtrate of *Bacillus subtilis* (AUBS 2) against mycelial growth and dry weight of *R. solani* (Poisoned food technique)

Tr. No.	Concentration of culture filtrate (%)	Solid medium assay		Liquid medium assay	
		Mycelial growth (mm)	Per cent inhibition over control	Mycelial dry weight (mg)	Per cent inhibition over control
T1	5	82.25	8.61	298.33	5.49
T2	10	48.36	46.26	223.65	29.15
T3	15	18.97	78.92	189.86	39.85
T4	20	08.54	90.51	75.22	76.17
T5	25	00.00	100.00	01.87	99.40
T6	Carbendazim 50% WP @ 0.1%	00.00	100.00	01.97	99.37
T7	Control	90.00	—	315.68	—
	S.Ed	1.12		1.01	
	CD (p=0.05)	2.54	—	2.32	—

Table-5. Effect of culture filtrate of *Bacillus subtilis* (AUBS 2) on the Sclerotial germination of *R. solani* (cavity slide method)

Tr. No.	Culture filtrate conc. (%) 48 h.	Sclerotial germination (%)	Per cent inhibition over control
1	5	86.43	4.65
2	10	57.66	36.39
3	15	41.12	54.63
4	20	6.79	92.50
5	25	0.00	100.00
6	Carbendazim 50%WP @ 0.1%	0.00	100.00
7	Control	90.65	—
	S.EdC		
	D (p=0.05)	0.872.63	—

of seven days old culture. The flasks were incubated for 15 days at room temperature at 28 ± 2°C and filtered thereafter through filter paper Whatman No. 42 under vacuum. The mycelial mat was dried in hot air oven at 60°C until constant weight obtained. The mycelial dry weight in (mg) was recorded.

Sclerotial germination assay (Macko et al.[9]):

Different concentration of the culture filtrate of *B. subtilis* (AUBS2) @ 5, 10, 15, 20 and 25 per cent and sclerotia of test fungus (10 No.) were mixed in cavity slide and incubated for 48h. In Petri plate glass bridge moist chamber at (28 ± 2ºC). Carbendazim 0.1 per cent conc. was used for comparison. Cavity slides with sterile distilled water having spore suspension were kept as control. Observations were taken from microscopic fields from each slide. The total number of sclerotia germinated under each microscopic field was recorded and germination percentage was calculated.

Morphological and Biochemical characterisation of native Bacillus isolates :

The results presented in table-1 & 2 revealed varying degrees of cultural and biochemical characteristics of different *Bacillus* isolates. Based on the topography, shape, colour and colony characters of *Bacillus* spp,, the isolates were grouped into three categories. The first group identified as *Bacillus* spp. consisted of 10 isolates which are rod shaped, circular, opaque with serrated margins and creamy white. The second group had only three isolate which are rod shaped, circular, opaque, colony with serrated margin and Slightly yellow. The, third group included two isolates (AUBS 7 & AUBS 11) identified as rod shaped, round colony with smooth margin and white colour. Similar findings were also reported earlier by Lu *et al.,*[8] who reported that *B. subtilis* produced medium-sized colonies on Nutrient Agar medium that are grey-white, round, opaque and dry.

A total of 11 biochemical tests were

conducted to characterize the *Bacillus* spp. isolated from phylloplane of rice. All isolates were proved to be Gram positive. Positive results were obtained for Starch hydrolysis, Gelatin liquefaction Casein Hydrolysis, IAA production, HCN Production and Catalase test. Similarly, negative reaction was obtained for pigement test, Methyl red test, H_2S test and Urease utilization. These results are in accordance with Neha[12] where he characterised the *B. subtilis* based on morphological, physiological and biochemical studies. Rajashekhar *et al.,*[13] also characterised *B.subtilis* and *B. thuringiensis* based on morphological and biochemical studies.

Effect of Bacillus isolates against R. solani (Dual Culture technique) :

The results depicted in Table-3 revealed the efficacy of all the *Bacillus subtilis* isolates against *R. solani* when compared to control. However, among the different isolates tested, isolates AUBS 2 was found to be the most effective against *R. solani,* reducing the growth of the pathogen by 77.64 per cent when compared to control. This was followed by isolates AUBS13. The least growth inhibition of the pathogen was exhibited by isolates AUBS 4 (12.98%). Variation in inhibitory effect and rapid growth of bacterial antagonists on PDA medium strongly suggests that competition for nutrition and production capacity of antimicrobial components are the major mode of varying action of bacterial antagonist in the inhibition of test pathogen. Our study clearly demonstrated that the isolate *Bacillus subtilis* (AUBS 2) was highly inhibitory to the test pathogen (Table-3). Hence, isolate AUBS 2 was selected for the subsequent studies.

The results of the present study correspond with Sanjeevkumar[17] reported that *B. subtilis* inhibited the mycelial growth of a range of fungi causing seedling blight. Heterotrophic rhizobacteria of *Bacillus* sp. have been successfully used for biological control of several plant pathogens[1,15]. Malleswari[10] who stated that *B. subtilis* strain Cf 60 was very effective against *M. phaseolina* in dual culture technique. Neha[12] reported that *Bacillus subtilis* inhibited *R. solani* under *in vitro*. All these earlier reports are in line with the present findings.

Effect of culture filtrate of Bacillus subtilis (AUBS 2) against mycelial growth, dry weight and Sclerotial germination of R. solani :

The results revealed an increasing trend in the per cent inhibition with an increase in the conc. of culture filtrates of *B. subtilis*. The pathogen and amended with culture filtrate of *B. subtilis* recorded significant reduction in the mycelial dry weight, mycelial growth and sclerotial germination (Table 4&5). The flasks inoculated with pathogen and amended with culture filtrate of *B. subtilis* recorded significant reduction in the mycelial dry weight whereas, the flasks inoculated with *R. solani* alone (control) recorded the maximum mycelial dry weight (315.68 mg). The minimum mycelial dry weight (1.87 mg) of *R. solani* was recorded in 25 per cent conc. of the culture filtrate of *B. subtilis.* In solid media, Among

the various concentrations of culture filtrate tested, *B. subtilis* at 25 per cent conc. completely inhibited the mycelial growth of *R.solani* which was statistically on par with carbendazim 50% WP @ 0.1%. (table-4). Krishna and Pande[6] made similar such observations. Sanjeevkumar *et al*.[18] reported that the mycelial growth and biomass production of *M. phaseolina* was strongly inhibited by *B. cereus*.

The effect of culture filtrates on the sclerotial germination of *R. solani* was studied and the results are summarized (Table-5). Among the various conc. of *B. subtilis* the culture filtrate tested, the sclerotial germination of *R. solani* was completely inhibited by 25 per cent conc. of the culture filtrate and it was found to be on par with carbendazim 50% WP @ 0.1 per cent at 48 h observations. The potential antagonistic PGPR's against plant pathogenic fungi and their inhibitory action on conidial germination of pathogens have been reported by several earlier workers[21,22]. These reports are in line and add support to the present findings.

References :

1. Afouda LCA, D Schultz, G Wolf and K Wydra (2012). *Int J of Biol Chem Sci 6*(6): 5068-5077.
2. Ahmad F, I Ahmad, and MS Khan (2005). *Turkish Journal of Biology 29*(1): 29-34.
3. Atlas RM, LC Parks and A Brown (1995). Laboratory manual if experimental microbiology. Mosby-year book Inc, St. Louis.
4. Borkar SG (2018). *CRC Press* pp 93-107
5. Eckford MD (1927). *American J Hyg 7:* 200-201.
6. Krishna Kishore K and S Pande (2005). *Australian Plant Pathology 34*(2): 261-264.
7. Lore, J.S., T.S. Thind, M.S Hunjan, and R.K. Goel, (2007). *Indian Phytopathology. 60*(3): 296-301.
8. Lu Z, W Guo, and C Liu (2018). *J Vet Med Sci. 80*(3): 427-433. doi:10.1292/jvms.16-0572.
9. Macko, V., W. Woodbury and M.A. Stahmannu. (1977). *Phytopathology. 58:* 1250-1252.
10. Malleswari D (2014). *International J of Current Microbiology and Applied Sciences 3*(5): 755-763.
11. Mew, T.W and A.M. Rosales, (1992). *Biological control of plant diseases.* 113-123.
12. Neha, (2015). Studies on the management of sheath blight of rice caused by *Rhizoctonia solani* (Kuhn). M.Sc. (Agri.) Thesis, Annamalai University India.
13. Rajashekhar M, E Shahanaz, and K Vinay (2017). *J Entomol. Zool. Stud. 5*(5): 581-588.
14. Reynolds MT, FR Falkiner, R Hardy, and CT Keane (1979). *Journal of medical microbiology 12*(3): 379-382.
15. Ruiz SE, AJ Cristobal, RA Reyes, SJ Tun, RA Garcia, and AJ Pacheco (2013). *Int J of Experimental Botany 83:* 45-47.
16. Ryu E (1940). *Kitasato Arch of Experim Medic 17:* 58-63.
17. Sanjeevkumar K (2008) Studies on the management of banana wilt caused by *Fusarium oxysporum* f. sp. *cubense* (E.F. Sumith) Snyder and Hansen. Ph.D Thesis, Annamalai University India.

18. Sanjeevkumar K, P Balabaskar, T Sivakumar, R Kannan and KR Saravanan (2020). *Plant archives. 20*(1): pp 1547-1550.
19. Savary S, PS Teng, L Willocquat, and FW Jr. Nutter (2006). *Annual Review of Phytopathology 44:* 89-112.
20. Savary, S., F. Horagan, L. Willocqueet, K.L. Heong, (2012). *Crop Protection. 32:* 54e63.
21. Sivasakthi S, G Usharani and P Saranraj (2014). *A review 9*(16): 1265-1277.
22. Toua, Messaoud Benchabane, Fathia Bensaid and Rabah Bakour (2013). Evaluation of *Pseudomonas fluorscens* for the biocontrol of Fusarium wilt in tomato and flax. ISSN:
23. Vincent JH (1927). *Nature 7:* 159-165.
24. Wei G, JW Kloepper, and S Tuzun (1996). *Phytopathology 86:* 221-224.