International Archive of Applied Sciences and Technology
Int. Arch. App. Sci. Technol; Vol 10 [4] December 2019 : 148-153

[ISO9001: 2008 Certified Organization]
www.soeagra.com/iaast.html

IAAST
ONLINE ISSN 2277- 1565
PRINT ISSN 0976 - 4828

CODEN: IAASCA ORIGINAL ARTICLE

DOI: .10.15515/iaast.0976-4828.10.4.148153

Biological control of sheath blight of rice caused by *Rhizoctonia solani* Kuhn using marine associated *Bacillus subtilis*

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ABSTRACT

Rice is an important food crop, being the staple of more than half of the world's population. It is grown in tropical and subtropical regions of the world. This crop is subjected to be infected by several fungal, bacterial and viral diseases. Among the fungal diseases, sheath blight caused by Rhizoctonia solani is a serious disease which cause severe yield loss. Eventhough chemicals control the rice sheath blight disease, the use of continuous, inappropriate and non-discriminative chemicals is an agent to bring undesirable effect such as residual toxicity, development of resistance, ecological degradation, perilous to the wellbeing of humans and animals and increases the expense for plant protection. In this scenario, the present work has been aimed to isolate antagonistic rhizobacteria from the least explored coastal sand dune ecosystem, characterize their biological control potential for the suppression of R. solani and evaluate them in vitro and green house study. The fungitoxic effect of 15 isolates of bacterial biocontrol agents from various seaweed, sea water and sediments were evaluated under in vitro conditions on growth of Rhizoctonia solani, which is one of the causal agents of sheath blight. Bacillus subtilis (Accession number MK370673) Bs-1 was the most successful, showing 60.20% discretion of colony growth with a minimum mean mycelial dry weight (120.75 mg/50m/broth) of the pathogen. The present study identified that the usefulness of bacterial biocontrol agents against fungal pathogens is due to larger levels and early accretion of phenolics and phytoalexins, and the field study proved that, R. solani can be controlled by the usage of Bacillus subtilis.

Key words: *Rhizoctonia solani, Bacillus subtilis, Antifungal compounds, Rice.*

Received 20.04.2019 Revised 21.05.2019 Accepted 03.06.2019

CITATION OF THIS ARTICLE

T. Suthin Raj, A. Muthukumar, P. Renganathan, R. Sudha Raja Kumar and H. Ann Suji. Biological control of sheath blight of rice caused by *Rhizoctonia solani* Kuhn using marine associated *Bacillus subtilis*. Int. Arch. App. Sci. Technol; Vol 10 [4] December 2019 : 148-153

INTRODUCTION

Sheath blight of rice caused by a soil borne necrotrophic fungus *Rhizoctonia solani* Kuhn. It is regarded as one of the most widely distributed diseases of rice. Nowadays a major barrier to rice cultivation is the rice sheath blight disease by *Rhizoctonia solani.* [13]. Rice sheath blight disease has been reported to cause approximately 50% yield reduction in test plots of susceptible cultivars. Depending upon the age of the plant, time of infection and severity, it causes yield loss to the extent of 5.9 to 69 per cent [12]. Sclerotia may be uneven to spherical and measure 4-5 mm in diameter, basidia and basidiospores are formed under normal conditions and measure 10-15 x 7-9 nm and 8-11 x 6.5 nm respectively. Roy ([13] indicated that *R. solani* inhabits organic matter in the soil as mycelium because of its plant pathogenic activity and its saprophytic nature. Srinivas *et al.*, [14] stated that, a total crop loss varies from 30 to 40 percent in prevalent areas and it extend to a total loss when it spreads to upper parts of the plant and panicles is seen because of rice sheath blight

disease. This is usually encountered by the usage of fungicides with a wide range of activity that targets more than one disease. Presently, sheath blight disease management is done using systemic fungicides [3, 15, 16] and the bacterial bio-control representatives similar to plant growth enhancing rhizobacteria (PGPR). Several marine bacteria isolated from coastal sea water were also found to have antagonistic activity against *R. solani* [7]. However, the haphazard use of fungicides paves way to residual toxicity on the manufacture, development of chemicals resistance and also acts as an cause for environmental pollution and hence, there is an urgent need to develop alternative disease control procedures. Carling *et al.*, [2] and Suthin Raj *et al.*, [16] stated that a viable substitute to the use of chemical pesticide is felicitated by organic control of plant pathogens.

MATERIAL AND METHODS

Isolation, maintenance and identification of *R. solani*

The plants with representative signs of sheath blight disease were collected fresh from twenty traditional rice growing areas of Tamilnadu. The pathogens secluded from each of these localities formed one isolate of *R.solani.* The pathogen was isolated to potato dextrose agar (PDA) medium from diseased plants showing characteristic symptoms. The piece of the fruit with diseased symptoms was cut into small pieces, surface sterilized in 0.1% mercuricchloride solution for 30 seconds and then cleaned repeatedly with sterile distilled water and plated onto sterile PDA medium in 9 cm Petri dishes. The plates were incubated at room temperature (28 ±2°C) for five days and then checked for fungal growth. Rangaswamy, [9] had found the use of single spore isolation technique be used to obtain a clean culture.

Evaluation of antagonistic bacteria against *R.solani.*

Isolation of bacteria from seawater and sediments [4]

In the present study, Different serial dilutions such as, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were prepared from the 10ml made-up samples of seawater and sediments and as well from 10ml of seawater samples. For each dilution, 100µl wash down was spread on to petri plates containing approximately 15 ml of 1.5% ZoBell marine agar. The plates were then incubated at 25±2°C and bacterial colonies with different morphology were selected up every 6h up to 4 days and marked on the fresh plates with ZoBell marine agar. Pure cultures of each isolates were long-established by subsequent restreaking. Then, they were chosen with unique codes and stored in glycerol suspension (glycerol/bacterial broth of 1:1 v/v) in Eppendorf tubes at -80°C for further analysis.

Table A. List of bacterium recorded from the samples of seawater and sediments

S.No	Place	Bacterium	
		Seawater	Sediments
1	Samiyarpettai	*Bacillus cereus* *Aeromonas salmonicida* *Bacillus amyloliquefaciens*	*Lactobacillus fermenti*
2	Parangipettai	*Bacillus subtilis*	*Lysinibacillus fusiformis*
3	Cuddalore	*Aeromonas salmonicida*	*Aeromonas salmonicida*
4	Thondi	*Lactobacillus fermenti*	*Aeromonas hydrophila*
5	Rameshwaram	*Bacillus subtilis* *Aeromonas salmonicida*	*Bacillus subtilis* *Aeromonas hydrophila* *Aeromonas salmonicida*

PCR amplification of fungal ITS region from *Bacillus subtillis* isolates

The primers FIGS 1(Forward) and FIGS 2 (Reverse) were used to amplify the *Bacillus velezensis* species in different soil samples.

FIGS 1 - 5'- GTA AGC CGT CCT TCG CCT CG - 3'

FIGS 2 - 5'- GCC ATA CTA TTG AAT TTT GC - 3'

The cocktail for the amplification was prepared in 0.2 ml PCR tubes as detailed below:
DNA 25 ng/ µl 2.00 µl

dNTPs (2.5 mM) 2.00 µl

Forward Primer (30 picomole) 2.00 µl

Reverse Primer (30 picomole) 2.00 µl

10x assay buffer 2.00 µl

Taq polymerase (3 units/ µl) 0.40 µl
Magnesium chloride 2.00 µl
Sterile distilled H20 8.60 µl
Total 20.00 µl

Then the 0.2 ml PCR tubes were placed on to a thermocycler (Agilent technologies) and the thermal cycler was programmed as follows:

Profile 1: 94 °C for 1 min Initial denaturation
Profile 2: 94 °C for 1min Denaturation
Profile 3: 58 °C for 1min Annealing
Profile 4: 72 °C for 1 min Extension
Profile 5: 72 °C for 5 min Final extension
Profile 6: 4 °C for infinity to hold the samples until attended

Profiles 2, 3 and 4 were programmed to run for 30 cycles. The amplified PCR products were run on 1.5% agarose gel in tris-borate buffer. The gel was stained with ethidium bromide, visualized on a UV-transilluminator and photographed in the gel documentation unit (Alpha Innotech Corp, USA).

Screening of marine bacterial isolates for antibiotic production

According to the morphological, Gram's discoloration and biochemical individuality described in the Bergey's manual, 26 strains having up to 5 bacterial category were enunciated from different samples like seaweed, sediment and seawater and were assessed for antibiotics production. Bacteria grown-up in the medium developing reserve zone around the discs were measured as antibiotic producers. Thus 3 bacteria from 16 strains were observed as antibiotic producers and they were then taken up for further viewing against plant pathogens.

Dual culture

B. subtilis was developed on nutrient agar (peptone -5g, meat extract – 1g, yeast extract 2g, sodium chloride- 5g, pH 7.0) medium. An 8 mm vigorously growing PDA culture disc of the pathogen was set aside on PDA medium in a sterilized petri dish at one side, 1.5 cm away from the edge of the plate, and incubated at a temperature of 28 ± 2°C. After forty eight hrs, actively growing 48-h-old cultures of the respective experimental bacteria were individually noticed on to average at the contrary side of the plate, 1.5 cm away from the edge of the plate. And at room temperature of (28 ± 2°C) the inoculated plates were incubated. Three replications were maintained for antagonist activity. Potato dextrose agar medium (PDA Medium) inoculated with the pathogen alone served as a control. After 8 days, the radial progress of the pathogen was seen and measured. The results were expressed as percent growth inhibition over control. The most effective isolates of *B. subtilis* were used for further study.

Mycelial dry weight

PDA was prepared in 250 ml Erlenmeyer flasks and autoclaved. Culture filtrates of *Bacillus subtilis* at 10 ml were added to 40 ml broth in flask so as to get a final concentration of 20 per cent of the filtrate in broth. The broth was inoculated with 8mm culture disc of *R. solani* and incubated for 10 days at 28± 1^0C. The control solution was the broth without the inclusion of filtrate. After the incubation period, on an earlier weighed filter paper, the mycelial mat was harvested and dried out at 105^0C for 12 h in a hot air oven and was cooled in desiccators. The mycelial weight was documented as mg/50 ml/broth.

Evaluation of *Bacillus subtilis* for the management of *R.solani* under field conditions

The field trials were conducted at Shathankudi Village, Perambalur-District between December 2017 and March 2018 in a field with a history of rice sheath blight incidence. Trials were set in plots (33 x 13feet) laid out in a randomized block design. Thirty days old rice seedlings of var. ADT 36 were transplanted in cement carriages. *R. solani* was inoculated over the plant canopy by one gram rice hull/rice grain, placed on basal leaves and closed with polythene bags on the 20^{th} day after transplanting. The below given treatment schedule was designed on the basis of the above phenomena. The cultivar ADT 36 was raised as per the Crop Production Guide (2017).

Treatment details

T_1: Application of *Bacillus subtilis* (seed treatment)
T_2: Application of *Bacillus subtilis* (prophylactic spray at 20, 40 and 60 DAT)
T_3: T_1 + T_2

T_4: Seed treatment with Hexaconazole + spraying 50 and 75 DAT
T_5: Control.

Disease Incidence

The evaluation of sheath blight damage for rice plant was visualized on their 30th, 50th and 70th days after transplantation. The strength of sheath blight was calculated as per cent disease index (PDI) grade chart given by Ravinder Reddy [10] and using the formula given by McKinney [8] as described earlier.

Plant Growth Parameters

Growth parameters *viz*., plant height, number of productive tillers, 1000 g weight, straw yield and grain yield were analyzed for the plants.

List of bacterial biocontrol isolate with their NCBI Accession numbers

S.No	Bio inoculants	Accession Number
1	*Bacillus subtillis*	MK370673
2	*Bacillus amyloliquefaciens*	MK368811

Experimental design and data analysis

The experiments were conducted by completely randomized design (CRD) with three replications. The significant difference, if any, among the means were compared by the Duncan's multiple range test (DMRT). Whenever necessary, the data were distorted before statistical analysis following appropriate methods.

RESULTS

Effect of Marine bacteria against *R.solani*

The results of the screening of five isolates of bacteria against *R. solani* on PDA plates are given in Table 1. Among the *Bacillus* sp isolates *B. subtilis* Bs-1 was found to be the most effective against the test pathogen showing 68.80 per cent reservation of colony growth and minimum mean mycelial growth of pathogen (120.75). It was further followed by isolated *Bacillus amyloliquefaciens* showing 68.55 per cent reservation and minimum mean mycelial growth (139.75) which were statistical on par with each other. A minimum growth inhibition (57.40) and minimum mycelial growth of the pathogen was found by the usage of the isolate *Lactobacillus ferment.* All the isolates significantly minimized the mycelial growth of the pathogen over the control.

Mycelial Growth

The mycelial growth of the pathogen was experimented against *B.subtilis* at 10, 20, 30 and 40 per cent concentrations. Among them, *B.subtilis* isolated was significantly plummeting the growth of mycelium at 208, 162, 84 and 29 mg/50ml broth respectively. It was followed by *Bacillus amyloliquefaciens* isolated with 226, 196, 101 and 36 mg/50ml broth. All the isolates significantly reduced the mycelia growth of the pathogen over the control (Table 1). Hence the superior isolate, *B.subtilis* was used for the then studies.

Effect of *B. subtilis* on incidence of sheath blight under field conditions

From the results (Table 2) it can be identified that the use of *B. subtilis* (seed treatment + prophylactic spray at 20, 40 and 60 DAT) (T_3) significantly has minimised the incidence of sheath blight at 30, 50 and 70 days after transplanting as compared to the other forms of treatments. This was followed by the treatment of Hexaconazole (seed treatment + prophylactic spraying 30 and 45 DAT) (T_4).

Effect of *B. subtilis* and Hexaconazole on growth and yield of *R. solani* under field condition

Table 3 pictures that all treatments have significantly improved the fruit yield and growth, as compared to the control. From the various groupings that was assessed it was found that, seed treatment + prophylactic spraying 30, 50 and 70 DAT with *B. subtilis* (T_3) considerably enhanced the mean plant height (96.40 cm), mean number of productive tillers (15 nos), mean 1000g weight (246g), straw yield (8.50 ton/ha) and grain yield (35 g/plant), in comparison to all other methods followed by spraying Hexaconazole (seed treatment +prophylactic spraying at 30 and 45 DAT) (T_4).

DISCUSSION

From various regions of Tamilnadu, five isolates of *B. subtilis* were isolated and tested their efficiency against *R.solani*. In the present study, it was found that among the ten isolates,

for *B. subtilis* Bs-1 maximum kept the growth of *R.solani* in dual plating technique. A same sort of result was found in the studies of Vivekananthan *et al.*, [17] and Abarna *et al.*, [1]. They have stated that, isolate Bs-1 has powerfully inhibited the growth of *R.solani* in laboratory circumstances and field situations. This may be because of *B. subtilis* isolates production of a collection of antifungal antibiotics such as 2, 4-diacetylphloglucinol, oligomycin, phenazine, pyoluteorin, pyrolnitrin and pyocyanin [5]. Hofte and Bakker, [6] and Reddy *et al.*, [11] had earlier stated that, antifungal compounds like HCN, salicyclic acid and 2-hydroxyl phenazine produced by bacterial biocontrol agents has suppressed the plant pathogenic fungi.

Table 1. Evaluation of various isolates of *Bacillus subtilis* against *R. solani* by dual culture technique

SI . No.	Isolates	Linear growth (mm)		% Growth inhibition	Mycelial dry weight (mg/50m/broth)				
		Antagonist	*R. solani*		10%	20%	30%	40%	Mean
1	*Bacillus subtilis*	62.00	28.00	68.80[a]	208.00	162.00	84.00	29.00	120.75[a]
2	*Bacillus amyloliquefaciens*	60.80	29.20	68.55[b]	226.00	196.00	101.00	36.00	139.75[b]
3	*Bacillus cereus*	58.60	31.40	65.11[b]	267.00	219.00	117.00	49.00	163.00[c]
4	*Aeromonas salmonicida*	55.50	35.50	61.66[c]	294.00	223.00	136.00	52.00	176.25[c]
5	*Lactobacillus fermenti*	51.70	38.30	57.40[d]	324.00	246.00	156.00	56.00	195.50[d]
6	Control			0.00[e]	480.00	480.00	480.00	480.00	480.00[e]

*****Values in the column followed by common letters do not differ significantly by DMRT (P=0.05)

Table 2. Effect of *Bacillus subtilis* on growth and yield attributes under greenhouse conditions

Treatments	Mean plant height (cm)	Mean no. of productive tillers	Mean 1000 g weight	Straw yield (ton/ha.)	Grain yield (g/plant)
T_1 - Application of *Bacillus subtilis* (Seed treatment)	83.00[c]	12[e]	18[d]	5.42[c]	26[d]
T_2 - Application of *Bacillus subtilis* (prophylactic spray at 30, 50 and 70 DAT)	70.40[e]	9[f]	15[d]	3.96[c]	21e
T_3 - T1 + T2	96.40[a]	15[a]	24[a]	8.50[a]	38[a]
T_4-Seed treatment with Hexaconazole + spraying, 30 and 45 DAT	95.10[a]	14[c]	23[a]	8.30[a]	35[b]
T_5- Control	74.00[d]	10[f]	13[e]	4.50[f]	18[e]

*****Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

Table 3. Effect of *Bacillus subtilis* on Sheath blight incidence under field condition

Treatments	Sheath blight incidence on 30th DAT	% Increase over control	Sheath blight incidence on 50th DAT	% Increase over control	Sheath blight incidence on 70th DAT	% Increase over control
T_1 - Application of *Bacillus subtilis* (Seed treatment)	4.1[c]	78	8.8[d]	78	12.6[d]	81
T_2 - Application of *Bacillus subtilis* (prophylactic spray at 30, 50 and 70 DAT)	4.9[b]	74	10.6[c]	73	15.2[c]	78
T_3 - T1 + T2	2.3[a]	88	6.6[a]	83	8.3[a]	88
T_4-Seed treatment with Hexaconazole + spraying, 30 and 45 DAT	2.6[a]	86	7.0[b]	82	10.2[b]	85
T_5- Control	7.5[d]		8.7[e]		9.2[e]	

*****Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

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