MANAGEMENT OF SEEDLING DISEASE OF CUCUMBER BY USING RHIZOSPHERE ANTAGONISTIC BACTERIA

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Accepted: 31 May, 2017

Abstract

A total of 47 bacteria were isolated from rhizosphere soil samples, collected from different locations of Bagerhat district in Bangladesh. Initially, all the isolates were tested for antagonism against virulent isolates of *Rhizoctonia solani* and *Sclerotium rolfsii* in dual plate culture, where 4 isolates were found to be antagonistic against both of the target fungi. Following a series of biochemical assays, all the antagonistic isolates were identified as *Bacillus* spp. However, in dual plate culture test, the antagonistic *Bacillus* isolates demonstrated a higher level of antagonism against *S. rolfsii* compared to *R. solani*. Interestingly, the thermo stable components of all *Bacillus* isolates completely inhibited the colony growth of both *S. rolfsii*. On the other hand, thethermo stable components *Bacillus* isolate AB4 showed the highest potentiality to inhibit mycelial growth of *R. solani*. In the pot experiment, the isolate AB4 also showed highest disease reduction (69.56 and 71.46%) against *R. solani* and *S. rolfsii*, respectively. In cucumber growth promotion test, it was found that all the *Bacillus* isolates significantly influenced plant height, root length, leaf area, fresh shoot weight, dry shoot weight, fresh root weight and dry root weight.

Key words: Rhizosphere, Antagonistic bacteria, Seedling disease, Cucumber

Introduction

Cucumber (*Cucumis sativus* L.) is one of the most important and widely cultivated vegetable crops in Bangladesh (Anon, 2011). It is grown in small gardens, large commercial farms and glasshouses. There is about 16,750 acres of land under cultivation of cucumber and the production is about 32,480 metric tons (Anon, 2011). The yield of cucumber is very low in our country compared to those other developing countries. There are many reason behind the lower yield of cucumber and among those, seedling disease caused by *Sclerotium rolfsii* and *Rhizoctonia solani* is one of the important factor that hindering the production of cucumber in Bangladesh. Different inorganic and synthetic organic compounds such as DDT is an example of a pesticide that is banned in Bangladesh, but that is still available on the market (Meisner, 2004) are traditionally being used to control seedling diseases of cucumber. However, the synthetic chemicals are known to have several hazardous effects such as heavy metal poisoning, development of pesticide resistance, pest resurgence (pest outbreak), and destruction of non-target organisms, environmental pollution and poisoning cases including accidental poisoning.

Such potentially hazardous effects of synthetic agrochemicals have lead scientists around the world to search for new alternatives for pest management. Exploitation of Rhizosphere bacteria is one of such alternatives. In many previous studies, such bacteria were found to be effective as bio-control option against different plant diseases (Sakalauskas *et al*., 2014). They suppress plant disease through different mechanisms, such as production of siderophores, antibiotics or bio surfactant, competition for niche or nutrients, induction of systemic resistance, etc.(Siddiqui *et al*., 2005; Nourozian *et al*., 2006). Therefore, this aimed to isolate the rhizosphere bacteria and to assess their antagonism against seedling disease of cucumber caused by *S. rolfsii* and *R. solani.*

Materials and Methods

Study site

The study was conducted in the laboratory and glasshouse of the Department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Salna, Gazipur 1706 during 2010.

Isolation and Characterization of bacteria

Six soil samples were collected from Bagerhat district, Bangladesh. Bacteria were isolated from

individual sample following the soil dilution plate technique. Isolates were purified following single colony purification of the colonies with different appearance (color and colony shape). The purified isolates were preserved temporarily in 15% glycerol solution at 4°C. Dual culture experiment was carried out to observe antifungal activity of bacterial isolates against the pathogenic isolates of *S. rolfsii* and *R. solani* on PDA plates. Those bacteria which developed inhibition zone on dual culture plate against the pathogenic fungus on were preserved in 10% LKB (20 g/l peptone, 1.5 g/l K_2HPO_4, 15 ml glycerol and 1.5 g/l $MgSO_4$) media with 10 g skim milk at -80°C. A series of biochemical test such as Gram staining, growth in CAG medium, growth at 7% NaCl, growth at pH 5.7 in PD broth, oxidase test, catalase test, gliding motility test and growth at 45°C were conducted to characterize the antagonistic bacterial strains. The selected four strains of antagonistic bacteria genera were identified the following Bergey's Manual of Determinative Bacteriology (Hort *et al*., 1994).

Dual Culture Assay

The study was conducted following CRD with three replicates. A 5 mm disc from the margin of 3 days old fungal culture was placed at more or less centre of the PDA plate. Thereafter, a loopful of overnight grown antagonistic bacterial isolates (each) 10^8 CFU/ml suspension was sketch in straight line at 30 mm apart from the fungal disc. All plates were incubated in the dark for 25°C until the mycelia of *S. rolfsii*a nd *R. solani* fully covered the control plates. The radius of the fungal colony towards and away from the bacterial colony was measured. Degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control. The percentage of inhibition was calculated using the equation suggested by Riungu *et al.,* 2008.

Thermo stable antifungal components of Bacillus sp.

Bacillus spp. were incubated individually in 250 ml flask containing 200 ml YP (yeast peptone) broth for 7 days at 25°C. Thereafter, supernatant was collected from the culture after 10 min. centrifuging at 15000 rpm. Required yeast, peptone and agar for preparing 200 ml YPA medium were added to 200 ml supernatant and then autoclaved at 121°C under 1.1 kg/cm² pressures for 20 minutes. Five millimeter block of *S. rolfsii* and *R. solani* was placed on the center of petri dish containing supernatant and incubated at 30°C for 72 hours. Growth of *S. rolfsii* and *R. solani* on YPA plates at 30°C for 72 hours was also observed to determine the presence of thermo stable components in supernatant and their antifungal capacity. Diameter of mycelia growth was measured in mm using a ruler.

Estimation of nitrogenase enzyme activity (acetylene reduction assay)

One milliliter of the culture was transferred into an air-tight 30 ml bottle containing 10 ml of Nfb semi-solid medium (5 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.2H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$ and 0.5 % bromothymol blue in 0.2 N KOH (2 ml), 1.64% Fe-EDTA solution (4 ml), 0.02 g yeast extract, 2 g agar). After the pellicle formation (72 h), the bottles to be injected with 5% (v/v) acetylene gas with simultaneous removal of the same volume of air. The bottles then incubated at 30°Cfor 24 h. 1 ml of gas to be withdrawn and transferred to 7 ml Vacutainer TW tubes. The ethylene gas produced to be assayed using G-300 gas chromatograph equipped with a FID and 1-m Porpak N column.

Preparation of inocula

Antagonistic bacteria were grown in Erlenmeyer flasks (250 mL) containing 150 mL of YP broth for overnight on a rotary shaker (150 r. p. m.) at 25°C. The cells in cultured bacterial broth were collected by centrifugation at 15000 rpm for 10 min. at 0°C and washed with 10 mM $MgSO_4$ (Barriuso *et al.,* 2008). The sediment of cells was suspended with 10 mM $MgSO_4$. Optical density (OD) of mixed solution was determined by using spectrophotometer at 620 nm. Then OD value was adjusted to OD_{620}= 0.08 which contain about 10^8 cells ml^{-1} (Han and Lee, 2005).

Inoculums of *S. rolfsii* and *R. solani* isolates were prepared on autoclaved moist wheat bran in 3 l Erlenmeyer flask. Before using wheat bran were soaked in water for 12 hours @ 2:1 (w/v). Then it was sterilized in autoclave at 121°C under 1.1 kg/cm² pressures for 40 minutes. Five-millimeter diameter mycelial discs were cut from the edge of three days old PDA cultures in petri dishes. Thirty to thirty five discs of *S. rolfsii* and *R. solani* were added to autoclave wheat bran in the flasks and incubated at 25 °C for 14 days. It was shaken

by hand at 2-3 days interval for proper colonization. The colonized wheat bran was air dried for 2 days and stored at 4 ˚C for further pot experiment.

Fungi inoculation

Inocula of *S. rolfsii* and *R. solani* were thoroughly mixed with sterilized soil at the rate of 20 g /kg soil 7 days before seed sowing. Pots were observed for inoculum establishment up to 7 days. After complete growth of pathogen in soil, the soil was again mixed thoroughly to get uniform spread of the inoculum.

Bacillus inoculation

In case of *Bacillus* isolates, for ensuring uniform mixing with soil 2 ml of bacterial suspension was increased twenty five times by mixing distilled water. Inoculums were applied to soil 24 hours before seed sowing.

Pot culture study

Efficacy of Bacillus isolates in reduction seedling disease caused by S. rolfsii and R. solani of cucumber

Plastic pots (10 x 5 cm size) were filled with 200 g pathogen inoculated soil. The Cucumber variety 'Shila' packet of Lal Teer Seed Co., Bangladesh was used in this experiment and was collected from the retailer of Joydebpur, Gazipur.

The treatments of the experiment were viz. T1 = *R. solani* (control), T2 = *S. rolfsii* (control), T3 = *Bacillus* sp. AB_1 + *R. solani*, T4 = *Bacillus* sp. AB_1 + *S. rolfsii*, T5 = *Bacillus* sp. AB_2 + *R. solani*, T6 = *Bacillus* sp. AB_2 + *S. rolfsii*, T7 = *Bacillus* sp. AB_3 + *R. solani*, T8 = *Bacillus* sp. AB_3 + *S. rolfsii*, T9 = *Bacillus* sp. AB_4 + *R. solani* and T10 = *Bacillus* sp. AB_4 + *S. rolfsii*.

Effect of Bacillus isolates on the growth of cucumber

Plastic pots (10 x 5 cm size) were filled with 200 g sterilized soil. The Cucumber variety 'Shila' packet of Lal Teer Seed Co., Bangladesh was used in this experiment and was collected from the retailer of Joydebpur, Gazipur.

The treatments of the experiment were viz. T_1 = Control (2 ml 10 mM MgSO4), T_2 = 2 ml *Bacillus* sp. AB_1, T_3 = 2 ml *Bacillus* sp. AB_2, T_4 = 2 ml *Bacillus* sp. AB_3 and T_5 = 2 ml *Bacillus* sp. AB_4. The data plant height, root length, leaf area, fresh shoot weight, dry shoot weight, fresh root weigh and dry root weight were measured 30 days after seed sowing.

Observations and data analysis

For *in vitro* studies, observations of radial mycelial growth reduction were calculated in relation to growth of the control: (dc – dt) * 100 /dc = percentage inhibition of radial mycelial growth where, dc is radial growth measurement of the pathogen in control and dt is radial growth of the pathogen in the presence of treatments of four *Bacillus* sp., individually.

For *in-vivo* studies, the statistical analysis of data was carried out using standard analysis of variance- ANOVA by using MSTAT-C software (Russel and Eisensmith, 1993). To determine the significance of the difference between the means of two treatments, least significant difference (LSD) was computed at the 5% probability level.

Results and Discussion

Isolation and Characterization of bacteria

A total 47 bacteria isolates were retrieved from six soil samples collected from different locations of Bagerhat district. Among those, 17 isolates were collected from the areal root zones of Sundarban area, 10 from the upper soil surface of Sundarban and rest were isolated from the Shrimp firm and cultivated fields of Bagerhat (Table 1). Of those, four isolates showed antagonism against *S. rolfsii* and *R. solani*on in dual culture plate (Table 1).

Table 1. Details about the antagonistic bacteria

Sl. no.	Soil sample identity	Location	No. of Bacterial Isolate	No. of Antagonistic Isolate
1	BT-004.1	Upper surface, Sundarban ,Bagerhat	10	1
2	BT-004.5	Areal root, Sundarban, Bagerhat	17	1
3	BT-002.1	Shrimp firm, Bagerhat	4	1
4	BT-002	Shrimp firm, Bagerhat	6	0
5	BT-001	Cultivation field, Bagerhat	7	1
6	BT-003	Cultivation field, Bagerhat	3	0
		Total	47	4

The biochemical and morphological characterization test results are shown in Table 2. Biochemical tests for *Bacillus* showed positive results for growth at 45°C, pH 5.7 while negative growth in 7% NaCl. The isolates showed irregular type colony and yellowish gray color of the colony except *Bacillus* sp. AB_1 which showed pinkish colony color. The organisms showed strong positive results in the gliding motility test and weakly positive results in the catalase test and negative in oxidase test. The same results of morphological and biochemical test of *Bacillus* spp. were observed by several investigators (Montealegre*et al*., 2003; Selvakumar*et al*., 2007; Majumdar*et al*., 2011).Based on the morphological and biochemical characterization the antagonistic strains were primarily identified as *Bacillus* spp.

Dual Culture Assay

The result of dual plate culture of *Bacillus* isolates against *S. rolfsii* and *R. solani* are presented in the Table 3 and Figures I and II. Every isolates of the *Bacillus* were found more effective in inhabiting the radial growth of *S. rolfsii*in comparison to *R. solani*. The highest 85.67 and 61.33% radial growth inhibition was observed by *Bacillus* isolate AB_4 in case of *S. rolfsii* and *R. solani*, respectively. The lowest 45.33 and 76.33% radial growth inhibition were observed on dual culture plates *Bacillus* isolate AB_1 against *S. rolfsii* and *R. solani*, respectively. Clear halo zone was observed on dual plates. This is the indication of antibiotic or toxic substance production by *Bacillus* sp. against *S. rolfsii* and *R. solani.* Mycelial color of both fungi were changed in interaction with *Bacillus* isolates. Mojica-Marín *et al*., (2008) observed that *Bacillus* sp. produce antifungal components to control plant pathogenic fungus.

Inhibition of mycelia growth of S. rolfsii and R. solani by Thermo stable antifungal components of Bacillus sp.

Bacillus strains released thermo stable components which significantly inhibited mycelial growth of *S. rolfsii* and *R. solani* (Table 4 and Fig. 3). *Bacillus* strains completely inhibited mycelia growth of *S. rolfsii* while 15 mm mycelial growth was recorded in control plates after 72 hours of incubation. In case of *R. solani*, the highest radial growth was observed control (40 mm) and the lowest was 3mm on supernatant of *Bacillus* AB_4.

Table 2. Morphological and biochemical characters of *Bacillus* isolates

Characteristic	*Bacillus* sp. AB_1	*Bacillus* sp. AB_2	*Bacillus* sp. AB_3	*Bacillus* sp. AB_4
Colony type	I	I	I	I
Colony color	P	YG	YG	YG
Gram staining	+	+	+	+
Growth in CAG medium	+	+	+	+
7% NaCl	-	-	-	-
pH 5.7	+	+	+	+
Growth at 45°c	+	+	+	+
Oxidase test	-	-	-	-
Catalase test	+(w)	+(w)	+(w)	+(w)
Gliding motility	+	+	+	+

Note: *I = Irregular; P = Pinkish; YG = Yellowish grey and *+ = Positive; + (w) = weakly positive; — =Negative

Table 3. Inhibition percent of radial growth of *S. rolfsii* and *R. solani* by *Bacillus* isolates in dual culture plate

Isolates	% Inhibition of radial growth	
	S. rolfsii	*R. solani*
Bacillussp AB_1	76.33 c	45.33 d
Bacillussp AB_2	81.93 b	50.67 c
Bacillussp AB_3	82.33 b	55.00 b
Bacillussp AB_4	85.67 a	61.33 a
CV (%)	4.68	5.32

Note: Values within a column with a common letter do not differ significantly (P= 0.05)

Table 4. Inhibition of mycelial growth of *S. rolfsii* and *R. solani* by Thermo stable antifungal components of *Bacillus strains*

Treatments	Colony diameter (mm)	
	S. rolfsii	*R. solani*
Control plate	15	40
Bacillus sp. AB_1	0	10
Bacillus sp. AB_2	0	7
Bacillus sp. AB_3	0	5
Bacillus sp. AB_4	0	3

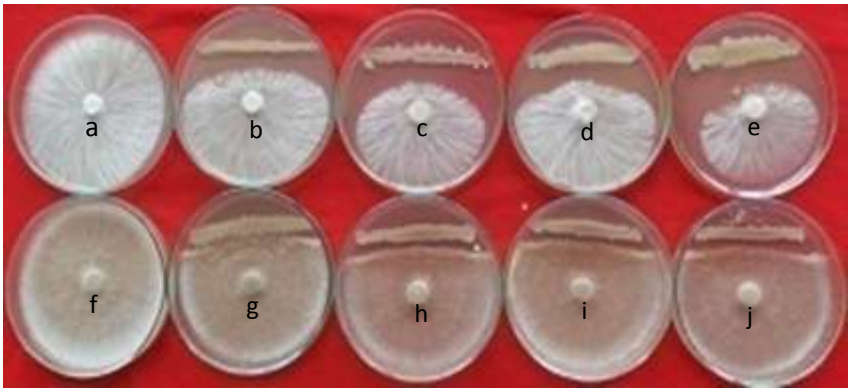

Fig.1. Inhibitin of growth of *S. rolfsii* and *R. solani* by *Bacillus isolates* three days after incubation

Note: (a) Control (*S. rolfsii*) (b) *Bacillus*sp AB_1 vs. *S. rolfsii* (c) *Bacillus*sp AB_2 vs. *S. rolfsii*(d) *Bacillus*sp AB_3 vs *S. rolfsii*(e) *Bacillus*sp AB_4 vs. *S. rolfsii*(f) Control (*R. solani*) (g) *Bacillussp* AB_1 vs. *R. solani*(h) *Bacillus*sp AB_2 vs. *R. solani*(i) *Bacillus*sp AB_3 vs. *R. solani*(j) *Bacillus*sp AB_4 vs. *R. solani*

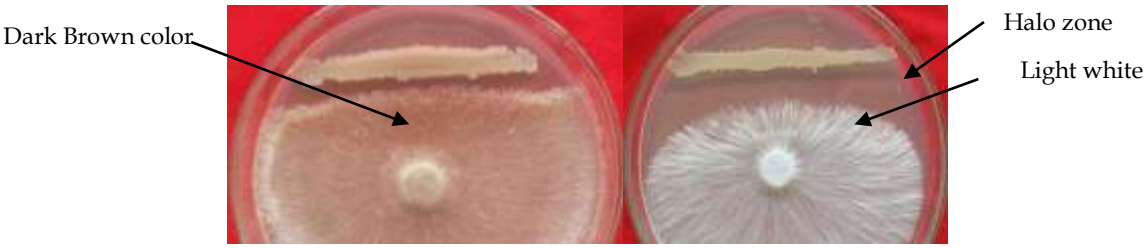

Fig. 2. Color change of *S. rolfsii* and *R. solani* mycelium in interaction with *Bacillus* strains and inhibitory halo zone developed by *Bacillus* strains

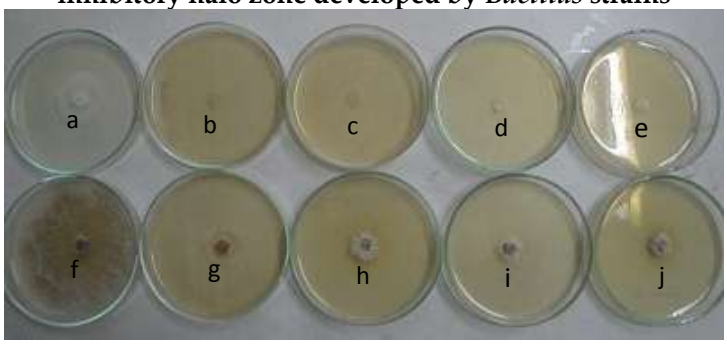

Fig. 3. Inhibition of mycelia growth by Thermo stable antifungal components

Note: (a) Control (*S. rolfsii*), (b) *Bacillus* p. AB_1 vs *S. rolfsii*, (c) *Bacillus* p. AB_2 vs *S. rolfsii*, (d) *Bacillus* p. AB_3 vs *S. rolfsii*, (e) *Bacillus* p. AB_4 vs *S. rolfsii*, (f) Control (*R. solani*), (g) *Bacillus* p. AB_1 vs *R. solani*, (h) *Bacillus* p. AB_2 vs *R. solani*, (i) *Bacillus* p. AB_3 vs *R. solani*, (j) *Bacillus* p. AB_4 vs *R. solani*

Estimation of nitrogenase enzyme activity (acetylene reduction assay)

Results of Nitrogenase activity (ethylene production) are showed in the Table 5. *Bacillus* sp. AB_1 and *Bacillus* sp. AB_4 showed nitrogenase activity (ethylene production) 0.89 nmolC2H4h-^{1} culture-^{1} and 1.46 nmolC2H4h-^{1}culture-^{1}, respectively under microaerobic conditions. But *Bacillus* sp. AB_2 and *Bacillus* sp. AB_3 did not show nitrogenase activity.

Table 5. Nitrogenase activity (ethylene production) by *Bacillus* isolates

Isolates	Nitrogenase activity (nmolC2H4h-^{1}culture-^{1})
Bacillus sp. AB1	0.89
Bacillus sp. AB2	0
Bacillus sp. AB3	0
Bacillus sp. AB4	1.46

Effect of Bacillus isolates on seedling mortality of cucumber

In pot experiment, application of *Bacillus* isolates significantly reduced seedling mortality caused by *R. solani* and *S. rolfsii*. The lowest seedling mortality 16.65 and 25.92% was observed *Bacillus* AB_4 treated *R. solani* and *S. rolfsii* inoculated pot, respectively. On the other hand, the highest seedling mortality 58.33% and 85.18% was observed in *R. solani* and *S. rolfsii* inoculated control pot, respectively (Fig. 4a.). *Bacillus* sp. AB_4 showed highest disease reduction 69.56 and 71.46% against *R. solani* and *S. rolfsii*, respectively (Fig. 4b.). The lowest disease reduction were observed 44.56 and 34.92% in *R. solani* and *S. rolfsii* inoculated pot, respectively when soil was treated by *Bacillus* sp. AB_1.

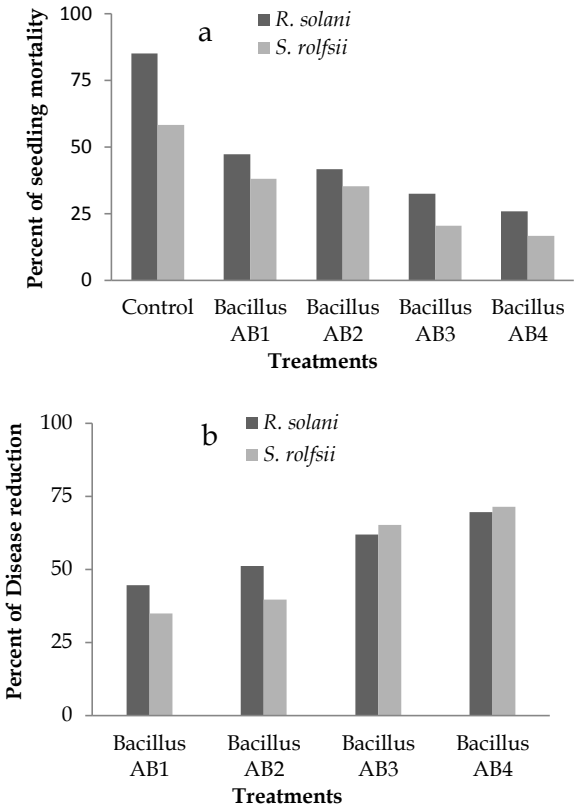

Fig. 4. Effect of soil application of Bacillus isolates on (a) seedling mortality and (b) of cucumber in *S. rolfsii* and *R. solani* inoculated pot

Effect of Bacillus isolates on growth promotion of cucumber

Bacillus isolates significantly influenced on the different plant growth parameters such as plant height, root length, leaf area fresh shoot weight, dry shoot weight, fresh root weight and dry root weight the height of cucumber. The highest plant height (33.65 cm), root length (21.71 cm), fresh

Table 6. Effects of *Bacillus* isolates on the growth of Cucumber

Treatment	Plant height (cm)	Root length (cm)	Leaf area (cm^2)	Fresh shoot weight (g)	Dry shoot weight (mg)	Fresh root weight (mg)	Dry root weight (mg)
Control (2 ml 10 mM MgSO4)	24.42f	17.2 f	63.81c	5.80d	37.20d	68.33d	3.30d
2 ml *Bacillus* sp. AB_1	33.65a	21.7 a	83.30a	8.53a	52.10a	86.00bc	4.93c
2 ml *Bacillus* sp. AB_2	32.33b	20.9ab	84.94a	8.20a	49.93b	97.33a	5.76a
2 ml *Bacillus* sp. AB_3	29.59c	19.00cd	75.72b	7.63bc	48.20c	84.00c	4.86c
2 ml *Bacillus* sp. AB_4	29.28cd	20.18bc	76.03b	8.07ab	49.50b	89.00b	5.13b
$LSD_{0.05}$	1.073	1.238	2.443	0.4752	1.717	4.234	0.174
CV (%)	0.0235	0.0411	0.0217	0.0412	0.0243	0.0327	0.0249

shoot weight (8.53 g) and dry shoot weight (52.10 mg) were observed in AB_1 treated pot. The highest leaf area (84.94 cm^2), fresh root weight (97.33 mg) and dry root weight (5.76 mg) were observed in AB_2 treated pot. The lowest growth of Cucumber seedling was observed in control plot where 2 ml 10 mM MgSO4 was mixed with soil.

Conclusion

From the collected six rhizosphere soil samples, 47 bacteria were isolated through soil dilution technique. Among them, four bacterial isolates showed antagonism against *S. rolfsii* and *R. solani* in *in-vitro* test on dual plate culture. These four antagonistic bacteria were identified as gram positive *Bacillus* through morphological and biochemical characterization. These four *Bacillus* isolates showed efficacy in controlling seedling disease of cucumber in pot experiment where *S. rolfsii* and *R. solani* were inoculated in soil. In another pot test, we observed that *Bacillus* isolates had also some growth promotion effect on cucumber seedling. As this experiment was conducted in controlled conditions, so further experimentation are required in field conditions to evaluate the efficacy of those *Bacillus* isolates against seedling disease of cucumber.

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