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Isolation of a potential antifungal *Baciilus subtilis* 37-JM07 strain from straw and its biocontrol efficacy to combat green mold disease of commercial mushroom, *Pleurotus ostreatus*

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ABSTRACT

Nine *Bacillus* strains were isolated from straw used in composting for mushroom cultivation and studied for their antifungal potential. The isolate 37-JM07 showed growth inhibition of all five phytopathogenic fungi used in spot inoculation method and it was identified as *Bacillus subtillis*. *In vitro* antagonism assay using single streak and double streak methods of dual culture technique has revealed the immense potential of the *B. subtilis* 37-JM07 with broad spectrum antifungal activity against 12 phytopathogenic fungi. In addition, the isolate 37-JM07 also could inhibit two gram positive bacteria in some extent. Cell free culture supernatant without concentration failed to suppress the growth of any fungi tested. *In vivo* field experiment resulted in 100% elimination of incidence of green mold disease in mushroom cultivation caused by *Trichoderma harzianum* due to treatment with *B. subtilis* 37-JM07. Moreover, about a 30% increase of mushroom yield over uninoculated control was also contributed by this potential *Bacillus* biocontrol agent.

INTRODUCTION

Despite the many achievements of modern agriculture, certain cultural practices have actually enhanced the destructive potential of diseases. These practices include use of genetically similar crop plants in continuous monoculture, use of plant cultivars susceptible to pathogens, and use of nitrogenous fertilizers at concentrations that enhance disease susceptibility. Plant disease control, therefore, has now become heavily dependent on fungicides to combat the wide variety of fungal diseases that threaten agricultural crops. Alternatives to many of the synthetic pesticides currently in use are needed, because they may lose their usefulness; due to revised safety regulations; concern over non-target effects; or due to the development of resistance in pathogen populations[28]. Thus, there is a need for new solutions to plant disease problems that provide effective control, while minimizing the negative consequences for human health and upon the environment[10].

Biological control based on microorganisms to suppress plant diseases offers a powerful alternative to synthetic chemicals. The abuse of chemical pesticides or fungicides to care or prevent plant diseases has caused soil pollution and detrimental ef-

fects in humans. It is desirable to replace chemical pesticides with materials that posses the criteria of high specificity against the targeted plant pathogen, easy degradability after effective usage, and low mass production cost. Biological control includes the use of natural or modified organisms, genes, gene products etc., to reduce the effects of undesirable organisms (pests, pathogens etc.), so as to favor desirable organisms such as crops, trees, animals, beneficial insects and micro organisms by their action of parasitoids, competition, induction of host resistance, predation, pathogenesis or antagonism.

Because of the increasing restriction in the use of chemical fungicides due to concern for the environment and human health, microbial inoculants have been experimented extensively during the last decade to control plant diseases[44]. As most of the soil and seed borne plant pathogens are fungi, biocontrol bacteria have been intensively investigated[31]. The use of bacteria has also been investigated mainly because the genetic and biochemical analysis of bacteria and the mass production of bacterial products are more straightforward than those of fungi, and thus the issue of bacterial control is expected to have great potential. *Agrobacterium, Pseudomonas, Bacillus, Alcaligenes, Streptomyces,* and others have been reported to be bacterial biocontrol agents[53, 17, 12].

Bacillus subtilis is antagonistic bacterial biological agent which control many airborne, seed borne and soil borne diseases of rice, wheat, sugarcane, jute, groundnut, cotton, rubber, soybean, tobacco, and vegetables etc. These bacteria can colonize in the root and leaf system of plants and compete and thereby suppress the growth of plant disease causing organisms. Antibiotics produced by antagonistic microorganisms have played an important role in biological control of plant diseases[30]. Members of the *B. subtilis* family produce a wide variety of antimicrobial substances like subtilosin A, subtilin, sublancin, Chitinase, and TasA are ribosomal antibiotics[7, 57, 37, 8, 51]; but others, such as chlorotetain, bacilysin, mycobacillin, difficidin, rhizocticins bacillaene, and lipopeptides including iturins, surfactins, and fengycins families are produced under the facilitation of ribosomal peptide synthetases of polyketide syntheses[3, 57, 56, 29, 38, 33, 39, 30]. In the lipopeptide family, iturins and fengycins display strong antifungal activity against a wide range of plant pathogens and are considered as the key factors for antagonism[15, 43].

Bacillus spp. have the characteristics of being widely distributed in soils, having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. Moreover, they are considered safe biological agents as they are non pathogenic, and their potential as biocontrol agents is considered to be high. However, the evaluation of bacteria has focused primarily on growth suppression[47], but the population dynamics and mechanisms of suppressing plant pathogens in soil by *Bacillus subtilis* have not been extensively investigated[28].

The present investigation hereby reports the isolation, identification and evaluation of a strain of *Bacillus subtilis* for its potential as biocontrol agent against green mold disease of mushroom, *Pleurotus ostreatus* caused by *Trichoderma harzianum*.

MATERIALS AND METHODS

Isolation and purification of bacterial strains antagonistic to other microbes: Straw used in compost preparation for commercial mushroom cultivation was collected from the National Mushroom Development and Extension Center, Savar, Dhaka. One gram straw was taken in a test tube containing 10 ml of sterile distilled water and mixed well by vortexing. This suspension was diluted by using serial dilution technique. The dilutions from 10^4, 10^5 and 10^6 were given a pretreatment with heat at 80 ^oC for 10 min to ensure that only heat resistant strains remained[5]. Then an aliquot of 0.1 to 0.2 ml from these pre-treated suspensions of isolation source was spread on to petri plates containing Nutrient Agar (NA) medium and the plates were incubated at 30 °C for 48 hrs. *Bacillus* colonies were picked from the medium based on colony morphology and sub cultured for several times to obtain pure culture.

Collection of phytopathogenic fungi and bacterial strains for antagonism assay: Twelve plant

TABLE 1 : The list of plant pathogenic fungi used in antagonism study

Pathogen's Name	Disease Name	Occurrence	Origin
Alternaria alternata	Leaf spot	Seed borne	Dhaka University
A. solani	Early blight	Seed borne	Jahangirnagar University
Botryodiplodia theobromae	Black Band	Seed borne	Dhaka University
Colletotrichum musae	Mango anthacnose	Soil borne	Dhaka University
Colletotrichum gloeosporioides	Die back/ Anthacnose	Soil borne	Dhaka University
Curvularia lunata	Grain mold, leaf spot	Seed & Soil borne	Dhaka University
Drechslera oryzae	Brown Rot	Seed borne	Jahangirnagar University
Fusarium moniliforme	Bakane or Fruit Rot	Seed borne	Dhaka University
Fusarium oxysporum	Fusarium wilt/Panama of Banana	Soil borne	Yamaguchi University, Japan
Sclerotium rolfsi	Root rot of ground nut	Soil borne	
Trichoderma harzianum	Green Mold	Soil borne	NMDEC, Savar
Trichoderma viridae	Green Mold	Soil borne	NMDEC, Savar

NMDEC= National mushroom development and extension center

TABLE 2 : List of bacteria used for *in vitro* antagonism assay

Name of the Bacteria	Source of collection
1. Staphylococcus aureus	Department of Microbiology, JU
2. Salmonella typhi	Department of Botany, JU
3. Shigella flexneri	Department of Microbiology, JU
4. Vibrio cholera	Department of Botany, JU
5. Escherichia coli	Department of Microbiology, JU
6. Bacillus subtilis	Department of Microbiology, JU
7. Bacillus mycoides	Department of Botany, JU
8. Pseudomonas sp.	Department of Microbiology, JU
9. *Vibrio parahaemolyticus*	Department of Botany, JU
10. *Escherichia coli* ATCC 25922	Department of Botany, JU
11. *Staphylococcus aureus* ATCC 25923	Department of Botany, JU
12. *Proteus mirabilis*	Department of Botany, JU

JU= jahangirnagar university; DU= university of dhaka

pathogenic fungi and twelve bacterial strains (TABLE 1 and 2) were collected from Culture Collection of Lab of Microbiology and Lab of Plant Pathology, Department of Botany; and Department of Microbiology, Jahangirnagar University; Plant Pathology Lab, University of Dhaka, Bangladesh; and from the National Mushroom Development and Extension Center, Savar, Dhaka, Bangladesh.

Preliminary Screening of bacterial isolates for antifungal activity: Selection of antagonistic bacterial isolates was done by employing spot inoculation method where a PDA (potato dextrose agar) plate (90 mm in diameter) was inoculated with pathogenic fungi at the center and 3 to 4 bacterial isolates at the periphery of same plate at a distance of about 15-20 mm from the center. Plates were incubated at 28 °C and observed for any clear zone around the bacterial isolate as inhibition of fungal growth till 10 days of inoculation. The fungal pathogens used in this primary screening of antifungal activity of bacterial isolates were *Alternaria alternata*, *Colletotrichum gloeospiroides*, *Drechslera oryzae*, *Fusarium oxysporum*, and *Trichoderma harzianum*.

Characterization of antifungal bacterial isolate: Selected antifungal bacterial isolates were characterized by morphological, physiological and biochemical tests. Colony morphology of selected isolates was examined by grown on nutrient agar for 2-4 days. Cell's morphology including cell shape, size and width; motility of cells; presence, shape and

position of endospores; cell dilation due to the spore and Gram-stain reaction was studied microscopically. The tolerance range of temperature, pH and salinity for the growth of selected antifungal bacterial isolates was determined. Nutrient agar was used as the basal medium. Growth at 25 °C, 30°C, 35 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C was recorded after 2 to 7 days of incubation. Growth at pH 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 and 9.0 was determined in media adjusted to the appropriate pH with HCl or NaOH after incubation of 3 days at 35 °C. Growth in the presence of NaCl (3%, 5% and 7% w/v) was recorded after 2-7 days incubation at 35 °C.

Metabolic characteristics of the growing cells of isolates such as Voges-Proskauer test; oxidase production, catalase production and urease production; utilization of citrate as sole carbon source; hydrolysis of casein, starch and gelatin; indole production; nitrate reduction; phenylalanine deamination/hydrolysis; H_2S production; and utilization of different carbohydrate sources such as glucose and lactose, and acid formation from glucose were carried out.

Identification: Selected bacterial isolates showing growth inhibitory activity to the test fungi in preliminary antagonism assay were identified according to the Bergey's Manual of Determinative Bacteriology[21] and Bergey's Manual of Systematic Bacteriology[52] based on morphological and biochemical characteristics determined. Identification of these unknown antifungal bacterial isolates from genus to species was carried out by matching experimental results with the identification key for strain identification as described by Priest (1993) and Slepecky and Hemphil (2006).

In vitro antagonism assay against plant pathogenic fungi and bacteria: The dual culture technique following both single streak and double streak methods was used to determine the growth inhibition potential of the selected bacterial isolate against 12 plant pathogenic fungi (TABLE 1). Pure cultures of these fungi were initially grown in Petri dishes containing PDA medium and incubated at 28±2 °C for 5 days. Then about a 4×4 mm agar block was cut from the edge of actively growing colonies of each fungus and placed on the center of another PDA plate (90mm). Antagonistic bacterial isolate was streaked toward the periphery at a distance of about 20-25 mm from the center of the same PDA plate. Then incubation was made at 28 °C for up to 7 days and the growth inhibition of test fungal pathogens by bacterial BCA was recorded.

The percent inhibition of radial growth of the test fungal pathogen by bacterial isolates was calculated by the following formula described by Sariah (1994) and Islam et al. (2009).

$$\text{Radial growth inhibition \%} = \frac{RC-RT}{RC} \times 100$$

Where, RC = Radius of test fungal growth in control plate without inoculation of any antagonistic bacteria (in mm).

RT = Radius of test fungal growth toward the streak of antagonistic bacterial isolate (in mm).

Overall growth inhibition efficiency of the antagonistic bacterial isolates was recorded by observation of radial growth of fungal mycelium, total mass of fungal mycelium, amount of spore formation by test fungi and colonization of isolate in the treatment plates compared with control plates.

The antibacterial activity of the selected potential bacterial biocontrol agent was also determined using cross-streak method. Single streak of selected bacterial biocontrol agent was made on surface of the nutrient agar. After that 12 bacterial strains (TABLE 2) were streaked at right angles to the original streak of bacterial biocontrol agent and incubated at 37 °C. The inhibition distance was observed and measured after 24-48 h. A control plate was also maintained without inoculating the isolated bacterial biocontrol strain to assess the normal growth of the bacteria.

In vitro antifungal assay with cell free culture supernatant of selected inhibitory bacterial isolate: *In vitro* antifungal assay with 48 h old cell free culture supernatant of the selected bacterial strain against all 12 phytopathogenic fungi was performed to check the action mechanism using agar well diffusion method. A loopful of 24 h old seed culture on nutrient agar slant was inoculated in 100 ml nutrient broth and incubated for 48 h at a temperature of 37 °C with agitation of 140 rpm in a shaker bath. The

culture supernatant was harvested by centrifugation at 8000 rpm for 5 minutes and filtered through 0.22 µm nitrocellulose membranes (Millipore Inc., USA). This filter sterilized culture supernatant of isolated bacterial biocontrol agent was used for application to the wells of potato dextrose agar plates. Two wells of 7.0 mm in diameter were made by using sterile agar borer and then 50 µl and 100 µl of the sterile supernatant prepared as per method described above was applied to the wells using micropipette. At the center of each treatment PDA plate the pathogen mycelia was inoculated separately for each pathogen. The plates were incubated at 25 °C and visual observation was made for growth inhibition of test fungal pathogens for three consecutive days of incubation. Control plate contained similar volume of filter sterilized culture medium without inoculation with BCA.

In Vivo field experiment to determine biocontrol potential: A field experiment was carried out in the National Mushroom Development and Extension Center, Savar, Dhaka to determine isolate's efficacy in controlling green mold disease of mushroom. Small substrate packets containing 500g of compost materials for spawning were used. 10 ml of *Bacillus subtilis* 37-JM07 and *Trichoderma harzianum* spore in sterile water was applied to spawn packet. Four different treatments with 20 replications used were as follows.

Treatment Composition

T-0 : Uninoculated control (nothing was added to the spawn packets of mushroom).

T-1 : 10 ml of spore suspension of *Trichoderma harzianum* at a concentration of 10^5/ml was injected into a pasteurized spawn packet after successful mushroom mycelium run through the opening cut for pin head emergence.

T-2 : 10 ml of *Bacillus subtilis* 37-JM07 suspension at a concentration of 10^5 cfu/ml was injected into a pasteurized spawn packet after successful mushroom mycelium run through the opening cut for pin head emergence.

T-3 : 5 ml of *Bacillus subtilis* 37-JM07 suspension of 10^{10} cfu/ml + 5 ml of spore suspension of *Trichoderma harzianum* at a concentration of 10^{10}/ml was injected into a pasteurized spawn packet after successful mushroom mycelium run through the opening cut for pin head emergence.

Bacillus inoculum preparation

The inoculum of isolated *Bacillus* was prepared according to a modified method of Ajilogba et al. (2013). Two or three loopfuls of selected bacterial isolate (BCA) from a 2-day old culture on nutrient agar (NA) were transferred to a 50 ml nutrient broth (NB) medium in 250 ml Erlenmeyer conical flask and incubated overnight at 30±1°C in a shaker bath with 120 strokes/min. Cells were harvested by centrifugation at 5000 rpm for 10 min which were then suspended in sterile water and adjusted to a concentration of 10^{10} and 10^5 cfu/ml.

Preparation of phytopathogenic fungi

Spore suspension of *Trichoderma harzianum* was used for artificial induction of green mold disease in spawn packets. The pathogen was grown on PDA for 21 days. The microconidial suspension of *Trichoderma harzianum* was prepared by pouring required amount of sterile water in each petri dish in order to separate the spores from the mycelium mass on agar medium and thus washed out and taken into a sterile beaker. The spore concentration was adjusted to the required concentration of 10^{10} and 10^5 spores/ml using heamocytometer. This suspension was then injected into spawn packets following a modified method of Adebayo and Ekpo (2005).

Preparation of spawn packets

Substrate was prepared by mixing sawdust and wheat bran at the ratio of 2:1. Calcium carbonate was used at the rate of 0.2%. The moisture level of the substrate mixture was adjusted approximately 65% by adding water. Polypropylene bags of 22.5×30 cm size were filled with 500 g of prepared mixture and packed tightly. The neck of the bag was prepared by using plastic heat resistant neck. A hole of about 2/3 of the volume of the bag was made with a sharp end stick at the center for space to put inoculums. The neck was plugged with cotton and covered with brown paper by placing a rubber band

to hold it in place. The spawn packets were then pasteurized through vapour. Inoculation with *Pleurotus ostreatus* was done by the mother spawn at the rate of two tea spoonfuls per packet. Then the inoculated packets were placed on racks of incubation room at a lower room temperature for mycelium run of mushroom. The spawn packets were cut open at opposite sides when the substrate was completely colonized with mycelium and transferred to culture room. Then the experiments were maintained at higher humidity of 80 – 85 % in culture room by regular watering till harvestment was completed.

Disease scoring and mushroom yield measurement

Observation was made for time of pin head emergence; colour, size and appearance of fruit bodies; disease incidence in all packets of all treatments. Harvestment was made from three flashes of fruiting. Harvestment from all spawn packets for individual treatment was scored by weighing fresh mushroom yields.

Disease incidence was recorded by counting the number of infected spawn packets and dividing it with the total number of packets used in each treatment. The result obtained was converted to percentage using the formula of Haruna et al. (2012).

Disease incidence = (Number of diseased spawn packets ÷ number of spawn packets assessed) × 100.

Percent increase in yield in treated bags over control (un-treated) was calculated using the formula:

$$\% \text{ Yield increase} = \frac{\text{Yield in treated packets - Yield in control spawn packets}}{\text{Yield in control spawn packets}} \times 100$$

RESULT AND DISCUSSION

Isolation and purification of antagonistic bacterial strains: A total of nine bacterial isolates were recovered from straw samples. All nine bacterial isolates were denoted as 37-JM01, 37-JM02, 37-JM03, 37-JM04, 37-JM07, 37-JM06, 37-JM07, 37-JM08 and 37-JM09.

Preliminary screening of bacterial isolates for antifungal activity

Nine purified bacterial isolates were screened for their efficacy in growth inhibition of five phytopathogenic fungi such as *Alternaria alternata, Colletotrichum gloeosporioides, Drechslera oryzae, Fusarium oxysporum,* and *Trichoderma harzianum* using spot inoculation method in a dual culture potato dextrose agar plate (Figure 1). Seven isolates showed their ability to inhibit the growth of one or more of 5 test pathogenic fungi. Isolates 37-JM01 and 37-JM04 did not show any activity against any of 5 test fungi in preliminary screening. However, the isolate 37-JM07 was found antagonistic to all 5 pathogens (TABLE 3).

TABLE 3 : Primary screening of possible *Bacillus* biocontrol agent based on growth inhibition in spot inoculation assay

Designated name of bacterial isolate	Antagonistic response of nine bacterial isolates against test fungal pathogen					% Pathogen antagonized by corresponding isolate
	Alternaria alternata	*Colletotrichum gloeosporioides*	*Drechslera oryzae*	*Fusarium oxysporum*	*Trichoderma harzianum*	
37-JM01	-	-	-	-	-	0
37-JM02	+	-	-	-	+	40
37-JM03	-	+	-	-	-	20
37-JM04	-	-	-	-	-	0
37-JM05	+	+	-	+	-	60
37-JM06	-	+	-	+	-	40
37-JM07	+	+	+	+	+	100
37-JM08	-	-	+	-	-	20
37-JM09	-	-	-	+	-	20

+ indicates inhibition; - indicates no inhibition

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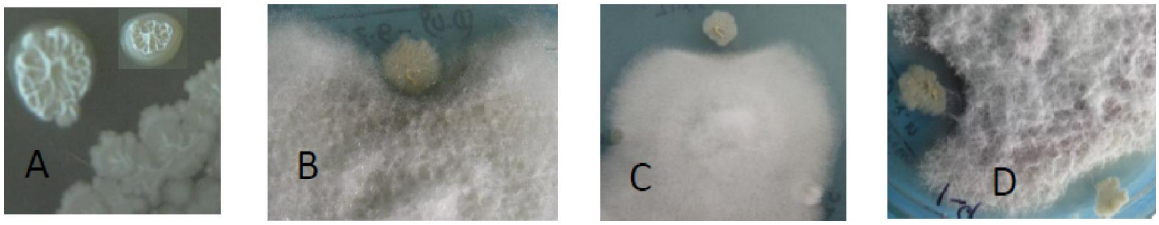
TABLE 4 : Characteristic features of the antifungal bacterial isolate 37-JM07

Response of bacterial isolate to the corresponding tests carried out					
Morphological characters		**Biochemical characters**			
		O_2 requirement	OA	Citate Utilization	+
Cell shape	Rod	Oxidase test	+	Growth at 6.5% NaCl	+
Gram positive cell	+	Catalase test	+	Growth at 40 ^oC	+
Spore formation	+	Gelatinase test	+	Growth at 45 ^oC	+
Central Spore	+	Urease test	-	Growth at 50 ^oC	+
Subterminal Spore	+	Nitrate Reduction	+	Growth at 55 ^oC	-
Ellipsoidal Spore	+	Phenylalanine hydrolysis	-	Growth at pH 5.0	-
Cylindrical Spore	+	Voges Proskaur test	+	Growth at pH 5.5	+
Spherical Spore	-	Indole test	-	Growth at pH 6.0	+
Cell dilation by spore	-	H_2S production test	-	Growth at pH 6.5	+
Cell width (≥ 1μ)	-	Starch hydrolysis	+	Growth at pH 7.0	+
Motility	+	Lactose Utilization	-	Growth at pH 8.0	+

'+' indicates positive response; '-' indicates negative response; OA= Obligately aerobic; FA= Facultatively aerobic



[From left: A, Single colony of isolate 36-JM07; B-D, Selection of antifungal isolate by spot inoculation]

Figure 1 : Screening of antagonistic *Bacillus* isolates by spot inoculation method

Identification of the antifungal bacterial isolate 37-JM07: Because of broad spectrum antagonism exhibited against all 5 fungal pathogens in preliminary screening the isolate 37-JM07 was characterized thoroughly (TABLE 4, Figure 1). The selected antagonistic bacterial isolate 37-JM07 was identified as *Bacillus subtilis* following the Dichotomous Key for Gram positive rods described in the Bergey's Manual of Determinative Bacteriology. Species identification was done based on spore shape and location, cell width, O_2 requirement, production of urease, production of H_2S, Voges-Proskaur Test, Citrate utilization, deamination of phenylalanine, and temperature and pH tolerance[41, 21, 48, 52]. The isolate 37-JM07 was motile and strictly aerobic with both catalase and oxidase activity. Thus it had been differentiated from strict anaerobes, such as *Clostridium*, *Desulfotomaculum*, *Sporohalobacter*, and *Syntrophospora*. Isolate 37-JM07 differed from *SporolactoBacillus*, which are facultative anaerobes without catalase but show scanty growth in air. It also did not belong to *AmphiBacillus*, a catalase-negative and oxidase-negative genus. Isolate grew well in the fermentation broth and having pH tolerance range of 5.5-8.0. Therefore, it did not belong to members of the genus *SulfidoBacillus*, which is nonmotile and grows optimally at a pH range of 1.9-2.4. All results summarized in TABLE 4 indicated that the unknown isolate 37-JM07 belonged to the genus *Bacillus*[19, 48, 52] and was identified as *Bacillus subtilis* because of its capacity for growth at pH ranging from 5.5 to 8.0; at 7.0% salinity; at temperature up to 50 ^oC but not at 55 ^oC; formation of single cylindrical and ellipsoidal spores but not spherical. Central or sub-central sporulation was spontaneously, not repressed by exposure to air and did not distend the cells.

In vitro antagonism assay of the selected *Bacillus subtilis* strain 37-JM07

Both single streak and double streak methods of dual culture were employed to assess growth inhibi-

TABLE 5 : Radial growth inhibition of 12 plant pathogenic fungi by *Bacillus subtilis* 37-JM07

Test fungal pathogens	Radial growth inhibition	
	Diameter (mm)	% Inhibition over control
Alternaria alternata	19.0 ± 1.0	42.22
Alternaria solani	22.33 ± 1.15	49.63
Botryodiplodia theobromae	16.67 ± 1.53	37.04
Colletotrichum gloeosporioides	35.0 ± 1.0	77.78
Colletotrichum musae	36.67 ± 0.58	81.48
Curvularia lunata	30.5 ± 0.50	67.78
Drechslera oryzae	35.33 ± 0.58	78.52
Fusarium moniliformae	30.0 ± 1.0	66.67
Fusarium oxysporum	31.0 ± 1.0	68.89
Sclerotium rolfsii	28.68 ± 0.58	63.70
Trichoderma harzianum	39.33 ± 0.58	87.41
T. viridae	37.33 ± 0.58	82.96

Values are average of 3 replications; ± stands for SD values

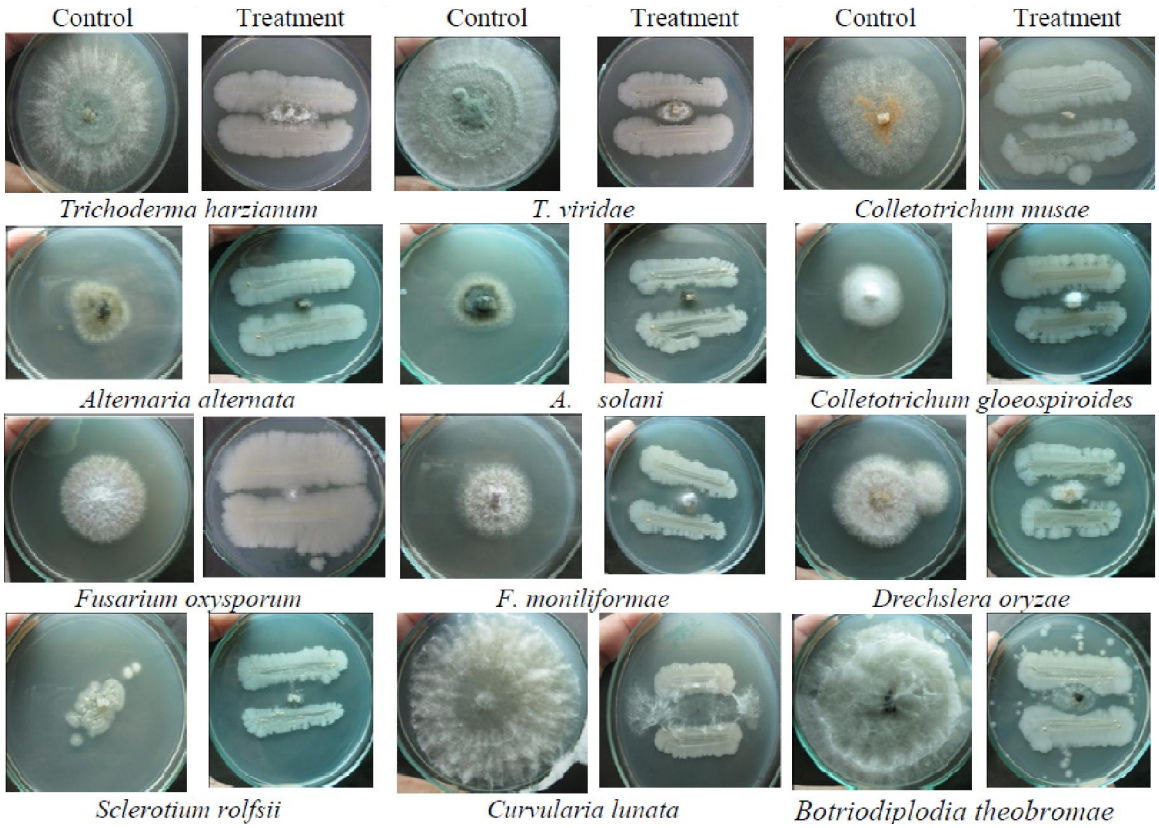


Figure 2 a : Growth inhibition of 12 plant pathogenic fungi by the treatment with *Bacillus subtilis* 37-JM07

tory efficiency of *Bacillus subtilis* 37-JM07. The isolate *B. subtilis* 37-JM07 could inhibit the radial growth of all 12 fungal pathogens ranging from the lowest of 37% over control in *Botryodiplodia theobromae* to the highest of 87% over control in case of *T. harzianum* (TABLE 5, Figure 2.b). The isolate contributed a growth inhibition of 60% over control in case of 75% of 12 test fungal pathogens and it was above 80% inhibition in case of 25% pathogens tested *viz.*, *Colletotrichum musae*, *trichoderma harzianum*, and *T. viridae*. Among the 12 bacteria assayed for growth inhibition by the se-

lected *B. subtilis* strain 37-JM07 only gram +ve *Staphylococcus aureus* and *Bacillus mycoides* were inhibited with clear zone of respectively 8.6 mm and 5.0 mm (Figure 2.c). Rest 10 bacteria did not show any response against the antagonistic isolate of *B. subtilis* 37-JM07. Growth inhibition of gram positive bacteria *Staphylococcus aureus* and *Bacillus mycoides* is the indication of antibiotic production by the *Bacillus subtilis* strain 37-JM07 which is also supported by many studies worldwide[34, 6].

Saha et al. (2012) reported two *Bacillus subtilis* strains with broad-spectrum activity against *Alternaria alternata, Colletotrichum gloeosporioides, Curvularia eragrostidis* and *Fusarium oxysporum* with percent growth inhibition ranging from 58 to 81. However, Ashwini and Srividya (2014) reported only 40 to 57% inhibition to these same fungi in their study from India. Similar findings were also reported by Furuya et al. (2011), Karimi et al. (2012), Ajilogba et al. (2013) and Zhang et al. (2012). *Bacillus subtilis* also was reported to successfully inhibit upto 93% radial growth of *F. oxysporum* and only 44% of *C. gloeosporioides*[22]. In another study Rahman et al. (2007) reported 100% radial growth inhibition of *Colletotrichum gloeosporioides*. 17 to 74% radial growth of *Trichoderma* by *Bacillus* isolates in Malaysia and Thailand have also been reported[46, 9].

The overall antagonism efficacy of the isolate *Bacillus subtilis* 37-JM07 has revealed that only radial growth inhibition can't always reflect the efficiency of a biocontrol agent. Overall performance of the isolate's antagonism considering factors such as inhibition zone, mycelium mass and sporulation by the fungal pathogen both in single and double streak method and colonization of *B. subtilis* 37-JM07 has resulted to a realistic conclusion. Inhibition potential based on overall performance has been grouped in to 3 categories *viz.,* above 90% inhibition, over 80% inhibition and below 80% inhibition (TABLE 6). The antifungal *B. subtilis* 37-JM07 scored best performer against *T. harzianum, T. viridae* and *Colletotrichum musae* for above 80% radial growth inhibition followed by *Drechslera oryzae,* C. *gloeosporioides* and *Fusarium* spp. for growth inhibition of 66-77% while lowest inhibition exerted on *Botryodiplodia* and *Alternaria* spp. with 37-49% inhibition over control. However, based on estimation of overall performance the isolate 37-JM07 has shown its highest effect on *Alternaria, Colletotrichum,* and *Trichoderma viridae* for above 90% growth inhibition. It was followed by above 80% inhibition in *Botryodiplodia, Drechslera* and *Fusarium* and below 80% in *Curvularia lunata*.

Khan (2013) stated that dual culture method may not be the best choice for initial antagonist screening because it eliminates host plant and environment factors and it is most likely to detect only direct antagonism by antibiosis. It is also much less time and resource demanding in comparison to screening strat-

TABLE 6 : Efficacy of broad-spectrum antifungal *Bacillus subtilis* isolate 37-JM07 against 12 phytopathogenic fungi

Test fungal pathogens	Overall growth inhibition by *Bacillus subtilis* 37-JM07
Alternaria alternata	+ + + + +
Alternaria solani	+ + + + +
Botryodiplodia theobromae	+ + + +
Colletotrichum gloeosporioides	+ + + + +
Colletotrichum musae	+ + + + +
Curvularia lunata	+ + +
Drechslera oryzae	+ + + +
Fusarium moniliformae	+ + + +
Fusarium oxysporum	+ + + +
Sclerotium rolfsii	+ + + +
Trichoderma harzianum	+ + + +
T. viridae	+ + + + +

+ – indicates inhibition; 5+ stands for >90% inhibition; 4+ for >80%; 3+ for <80% inhibition

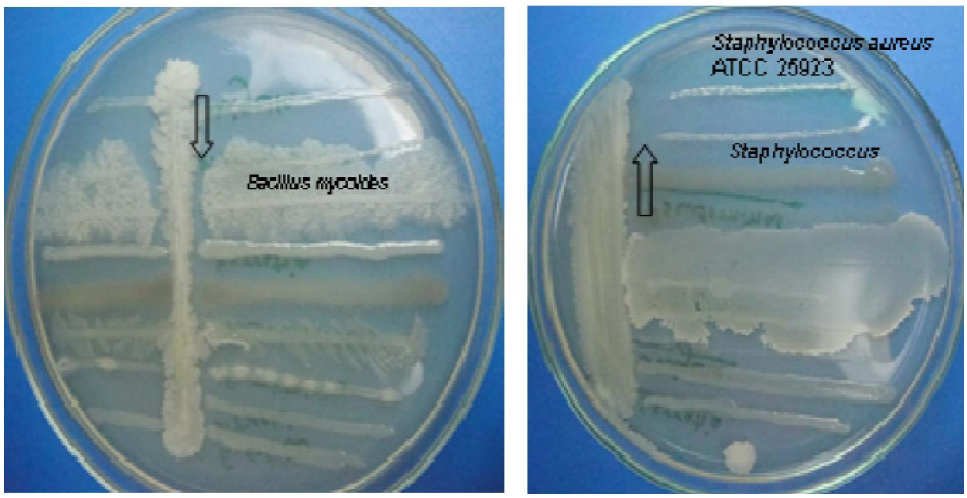
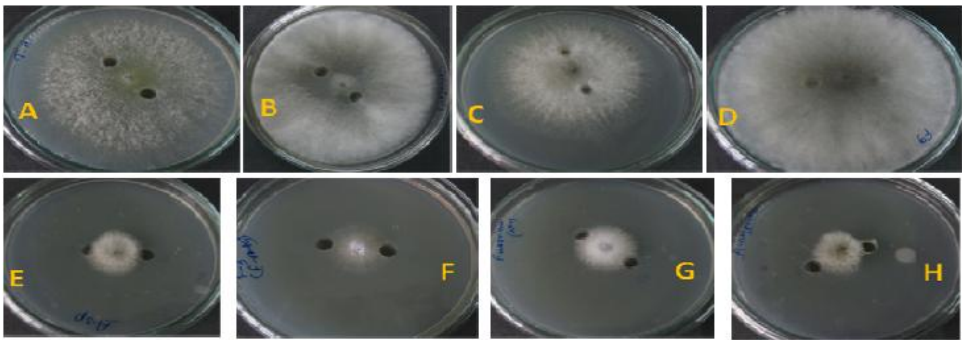


Figure 2b : Antibacterial activity of the isolated *Bacillus subtilis* strain 37-JM07



A = *Trichoderma harzianum*, B = *Curvularia lunata*, C = *T. viridae*, D = *Botryodiplodia theobromae*, E = *Alternaria solani*, F = *Fusarium oxysporum*, G = *F. moniliformae*, H = *A. alternata*

Figure 2c : *In vitro* growth inhibition assay with cell free culture supernatant of *Bacillus subtilis* 37-JM07 by agar diffusion method

egies involving more components, and when conducting a trial with large collections of bacteria and fungal pathogens of multiple host plant species, this method becomes a reasonable approach.

None of the pathogens were inhibited by culture supernatant of *B. subtilis* strain 37-JM07 (Figure 2.c). This result might be the indication either of insufficient concentration of antifungal compound secreted in the culture medium or absence of antifungal substances in the culture medium which are assumed to be induced only by pathogen attack.

The arrow indicates inhibition zone against *Bacillus mycoides* (left) and *Staphylococcus aureus* (ATCC- 25923) and *S. aureus* (local strain) (right)

Potential of *Bacillus* isolate 37-JM07 to combat green mold disease of mushroom: TABLE 7 represent the effect of selected isolate *Bacillus subtilis* 37-JM07 on the incidence of green mold and mushroom yield. 100% suppression of disease incidence was resulted by the treatment of *Bacillus* isolate 37-JM07 in the spawn packets amended with isolate and also in the packets amended with both pathogen and isolate. Highest yield was recorded in packets treated with isolate and lowest in the packets treated with both pathogen and isolate with average yield of 94g per spawn packet. Isolate 37-JM07 also increased mushroom yield up to 30% over untreated control. Nagy et al. (2012) reported 15-21% yield increase of oyster mushroom by the treatment of *B. amyloliquefaciens* which also overcome problem of green mold infection. Chittihunsa reported in 2007 that *Bacillus* isolates can significantly suppress the green mold infection by their treatment at a rate of 10^6cells/ml along with *Trichoderma* spores of 10^6/ml per spawn packet. Another study in Malaysia reported 6% to 25% yield increase over control caused by treatments of several *Bacillus* isolates[46].

The widely recognized mechanisms of biocontrol

TABLE 7 : Role of *Bacillus subtilis* 37-JM07 on the mushroom yield and green mold disease incidence

Treatment	Mushroom yield (g)	Yield increase over control (%)	Green mold disease incidence (%)
T-0: Non inoculated Control	72.7 ± 25.12	-	10
T-1: *Trichoderma* spore	0	-	100
T-2: *Bacillus subtilis*	94.25 ± 3.8	29.64	0
T-3: *Trichoderma* spore+ *B. subtilis*	92.8 ± 4.5	27.64	0

Data are average of 20 replications

action are competition for an ecological niche or substrate, as well as the production of inhibitory compounds and hydrolytic enzymes that are often active against a broad spectrum of fungal pathogens. Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens [18, 50]. *Bacillus* spp. in particular are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents[4, 50]. Moreover, they are spore-formers, which imparts a natural formulation advantage over other microorganisms[13, 43, 18]. Results of present investigation have been evident for the potential of *Bacillus subtilis* 37-JM07 strain to be used in commercial formulation for biological control of green mold of mushroom caused by *Trichoderma* spp. and other fungal diseases of crop plants[32].

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