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In Vitro Microbiological Control of *Alternaria macrospora*, a Leaf Spot Pathogen of Bt Cotton with *Bacillus subtilis* subsp. *inaquosorum* RLS52

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ABSTRACT

Alternaria leaf spot disease caused by *Alternaria macrospora* (Zimm.) is one of the most predominant disease of Bt cotton having national and regional significance. In present work a *Bacillus* species, namely *Bacillus subtilis* subsp. *inaquosorum* RLS52 isolated from rhizospheric niches of healthy Bt cotton plant, was screened against *Alternaria macrospora* by dual culture method. This *Bacillus* sp. significantly inhibited the radial mycelial growth of *Alternaria macrospora* i.e. 94.30 %. Upon finding the mechanism it was revealed that the *Bacillus subtilis* subsp. *inaquosorum* RLS52 produced diffusible non volatile metabolites, siderophore and chitinase enzyme to inhibit the growth of *Alternaria macrospora* during in vitro testing.

Key words: Bt cotton, *Alternaria macrospora*, *Bacillus subtilis* subsp. *inaquosorum* RLS52, Non volatile diffusible metabolites, Siderophore, Chitinase

Alternaria leaf spot disease of cotton is worldwide in distribution and reported from all cotton growing countries of the world (Hillocks 1991). In India, the Alternaria leaf spot disease of cotton is common and reported variable percent disease incidence from South Zones (10-35%), North Zone (7.33 to 31.83%) and Central Zone (9.66 to 31.30%). This disease was reported for the first time from Dharwad district of Karnataka and in Pune and Ahmednagar district of Maharashtra, India (Rane and Patel 1956). The Alternaria leaf blight of cotton essentially, a disease complex caused by two pathogens *Alternaria macrospora* and *Alternaria alternata* (Bashan *et al.* 1991, Bashan and Hernandez-Saavedra 1992). Considering the seriousness of the Alternaria leaf spot disease and to avoid yield losses as well as hazardous effect of fungicides, plant pathologist diverted their research to find out the bio-based, ecofriendly management system by using rhizospheric antagonistic microbes. Microbial control of plant diseases using rhizospheric antagonistic microorganisms could be a potential alternative approach for the management of Alternaria leaf spot of Bt cotton. There are some pieces of evidence, that *Pseudomonas sp.* (Gholve *et al.* 2014, Maurya *et al.* 2014, Kapadiya *et al.* 2015) *Bacillus sp.* (Abbo *et al.* 2014, Abdalla *et al.* 2014), *Trichoderma sp.* (Gveroska and Ziberoski 2012, Muthukumar and Venkatesh 2013, Rajput *et al.* 2013) were isolated from rhizosphere niches and found effective in controlling Alternaria leaf spot disease of cotton.

To find out the solution for the control of *Alternaria macrospora*, a leaf spot pathogen of Bt cotton, following objectives were set. i) To screen the *Bacillus subtilis* subsp. *inaquosorum* RLS52 against *Alternaria macrospora* by *in vitro* dual culture technique ii) To find out the mechanism involved in inhibiting the *Alternaria macrospora*.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were procured from M/S Hi-media, Mumbai and CDH Analytical / Guaranteed (AR/GR) grade chemicals and double glass-distilled water was used.

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Alternaria leaf spot pathogen of Bt cotton:

Alternaria macrospora, the phytopathogen of Bt cotton isolated during previous research work in the department of Microbiology Sant Tukaram College of Arts and science Parbhani was used in present research work.

Isolation of *Bacillus sp.*

The *Bacillus spp.* was isolated during previous work and identified as *Bacillus subtilis* subsp. *inaquosorum* RLS52 used in present research work.

Antifungal activity by dual culture method

Bacillus subtilis subsp. *inaquosorum* RLS52 was tested by using modified dual culture technique of Skidmore and Dickinson (1976) for antifungal activity against *Alternaria macrospora*. In this modified method, the PDA plates was inoculated with 5 mm fungal disc (5 day old culture) 1 cm away from the centre of the agar plate. Similarly 24 h old *Bacillus subtilis* subsp. *inaquosorum* RLS52 culture was inoculated in opposite direction, 1 cm away from the centre of the agar plate to maintain the equidistance of phytopathogen and antagonist from the centre of the agar plate. A control plate was kept without inoculation of *Bacillus subtilis* subsp. *inaquosorum* RLS52 and all the plates were incubated at room temperature for 6 days. The antifungal activity was determined by measuring the percent inhibition of radial growth (PIRG) (Skidmore and Dickinson 1976).

$$\% \text{ PIRG} = \frac{R_1 - R_2}{R_1} \times 100$$

Where,

R_1: radius of the fungal phytopathogen colony in the control plate

R_2: radius of the fungal phytopathogen colony in the direction of the antagonist colony

In vitro mechanism of microbial control agent

To reveal the biocontrol mechanism of microbial control agent, the *Bacillus subtilis* subsp. *inaquosorum* RLS52 was tested for the production of diffusible metabolites, siderophore production and cell wall degrading enzymes.

Detection of antifungal diffusible non-volatile metabolites

The diffusible antifungal non-volatile metabolite was detected by well diffusion assay (Schlumbaum *et al.* 1986). During screening *Bacillus subtilis* subsp. *inaquosorum* RLS52 was grown in Kings B broth at room temperature on a rotary shaker at 150 rpm for 36-48 h to obtain cell free culture filtrate. Potato dextrose agar plates were prepared and after solidification with the help of sterile cork borer (10 mm), a well was punched 1 cm apart from the centre on 90 mm diameter Petri plate. 5 mm plugs from leading edge of a 3-day old culture of *Alternaria macrospora* was punched and kept 1cm apart from the center and opposite to well in the PDA plate.

After incubation broth was centrifuged at 8000 rpm and filtered by using Millipore Syringe filter 0.22 µ (Hi-media) and well was loaded with 100 µl cell free culture filtrate aseptically. The control was kept without inoculation of rhizobacterial cell free culture filtrate. Both the plate control and test were incubated at room temperature for 5 to 6 days. After the incubation, the plates were observed for the zone of inhibition of radial growth and the % inhibition was calculated (Whipps 1987).

$$\text{PI} = \frac{R_1 - R_2}{R_1} \times 100$$

Where,

R_1: is radial growth of the pathogen in control plate (a control value)

R_2: is radial growth of the pathogen with antagonist (a test value)

Scanning electron microscopy study of co-culture

Similarly, both the *Bacillus subtilis subsp. inaquosorum* RLS52 and the phytopathogen, *Alternaria macrospora* were grown together in King's B broth as co – culture for 6 days to study the effect of secondary metabolites on the fungal culture. The effect of secondary metabolites on the phytopathogen was observed under the Scanning Electron Microscope at Sophisticated Instrumentation Center, Dr. Harisingh Gour Vishwavidyalaya, Sagar (M.P).

Siderophore production

The siderophore production was studied using modified succinate medium as per Meyer and Abdallah (1978), consisting (gm/l) Succinic acid (4), K_2HPO_4 (6), KH_2PO_4 (3), $(NH_4)_2SO_4$ (1), $MgSO_4$ (0.2), and pH (7.0). 0.1 ml of inoculum of *Bacillus subtilis* subsp. *inaquosorum* RLS52 was inoculated in 250 ml Erlenmeyer flask containing Succinate medium and then incubated on a rotary shaker incubator at room temperature for 48 h.

Detection of siderophore by liquid CAS assay

After incubation in Succinate broth, the supernatant was harvested by centrifuging the culture at 10,000 rpm in cooling centrifuge at 4°C for 10 min. The siderophore production was quantitatively detected as per Payne (1994) where 0.5 ml of cell free culture filtrate was mixed with 0.5 ml of CAS solution. A reference was prepared using the un-inoculated Succinate medium. The optical density of both the test and reference was measured at 630 nm and % siderophore units in the culture filtrate were calculated by using the following formula.

$$\% \text{ Siderophore Units} = \frac{Ar - As}{Ar} \times 100$$

Where,

Ar = Absorbance of reference at 630 nm

As = Absorbance of test sample at 630 nm

Detection of chitinase enzyme production

The chitinase productions ability of rhizospheric isolate *Bacillus subtilis* subsp. *inaquosorum* RLS52 was determined by using Colloidal Chitin Agar (Hsu and Lockwood 1975) amended with 0.4% colloidal chitin. The Colloidal Chitin Agar consists of 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g $MgSO_4$, 1g NH_4Cl, 0.5 g NaCl, 0.05 g yeast extract, 4 g colloidal chitin dissolved in 1000 ml distilled water. The pH of the medium was adjusted 7.0 and 20 g agar was added as a solidifying agent. The medium was sterilized at 121°C for

20 min and plates were prepared. Chitin agar plate was spot inoculated with bacterial suspension and incubated at room temperature for 4 days. The plates were observed for the zone of chitin hydrolysis around the spot inoculated culture.

RESULTS AND DISCUSSION

Antifungal activity by dual culture method

Rhizobacterial isolate *Bacillus subtilis* subsp. *inaquosorum* RLS52 found efficient in inhibiting *Alternaria macrospora* in dual culture method whose percent inhibition of radial growth was recorded as 94.30% (Plate 1, Table 1).

Table 1 Antifungal activity of rhizospheric isolate, *Bacillus subtilis* subsp. *inaquosorum* RLS52 against *Alternaria macrospora* by dual culture technique

Rhizospheric isolate	% of inhibition
Bacillus subtilis subsp. *naquosorum* RLS52	94.30

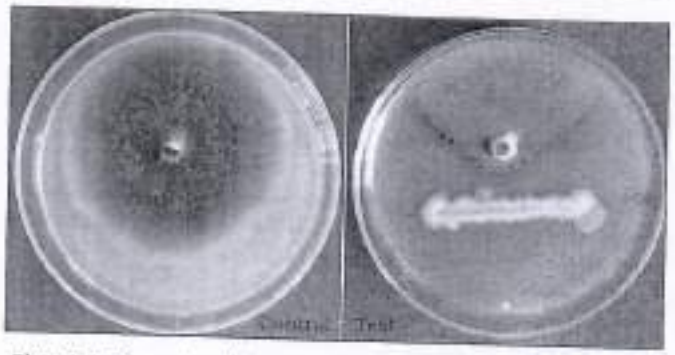

Plate 1 Antifungal activity of *Bacillus subtilis* subsp. *inaquosorum* RLS52 against *Alternaria macrospora* by dual culture technique

Earlier researchers had also exploited various microorganism such as *Trichoderma viride*, *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* (Baig *et al.* 2012), *T. harzianum*, *T. viride*, *T. virens* and *Aspergillus niger* (Reshu and Khan 2012), *Trichoderma* sp. and *P. fluorescens* (Muthukumar and Venkatesh 2015), *T. viride*, *T. viride* and *T. harzianum* (Rajput *et al.* 2013), in order to find control measure against *Alternaria* sp. in dual culture technique. Likewise, Abdalla *et al.* (2014) screened the *in vitro* antifungal potential of 27 antagonistic rhizospheric isolates belonging to genus *Bacillus* sp. against the phytopathogen *Alternaria alternata* causing early blight disease of Tomato plant. Four isolates were showed the inhibitory effect on the phytopathogen and the maximum antifungal potential exhibited by isolate B35 was (75.60%) against the phytopathogen.

The nine *Bacillus* isolates were isolated from mushroom composting straw and screened for antagonistic activity against plant pathogenic fungi *Alternaria alternata*. Among these nine isolates, one isolate *Bacillus subtilis* (37-JM07) showed 44.22 % growth inhibition of fungal phytopathogen *in vitro* antagonism (Akond *et al.* 2016). Tozlu *et al.* (2018) studied the antifungal activities of a total 13 candidate bioagent bacterial isolates of *Bacillus subtilis* (TV-6F, TV-12H, TV-17C and TV 125 A), *Bacillus megaterium* (TV 87 A and TV 91 C), *Bacillus pumilus* (TV 67 C), *Paenibacillus polymyxa* (TV 12E), *Pantoea agglomerans* (RK 92 and BRT-B), *Pseudomonas fluorescens* Biotip F (FDG 37), *Bacillus thuringiensis* subsp. *kurstakii* (BAB-410) and *Bacillus sphaericus* GC subgroup D (FD 49) and bioagent fungal isolates of *Trichoderma harzianum* (ET 4 and ET 14) against two isolates of *A. alternata* isolated from strawberry and cucumber on petri plate assays. This study reveals that *B. pumilus* TV 67C (87.63%-65.89%), *B. subtilis* TV 6F (77.61%-63.11%) and *B. megaterium* TV 87A (72.93%-68.87%) bacterial isolates were the most effective isolates against pathogenic fungi in *in vitro*. Our rhizobacterial isolate *Bacillus subtilis* subsp. *inaquosorum* RLS52 inhibited the *Alternaria macrospora* in dual culture up to 94.30% (Plate 1) which was greater than Abdalla *et al.* (2014), Tozlu *et al.* (2018).

Detection of antifungal diffusible non-volatile metabolites

The diffusible antifungal non-volatile metabolites production was tested by agar well diffusion assay (Schlumbaum *et al.* 1986). The Potato Dextrose Agar Plates inoculated with 5 mm mycelial disc at centre and agar well inoculated with cell free culture filtrate of rhizospheric isolates showed inhibition of fungal phytopathogen after 6 days of incubation.After six days incubation, the filtrate of *Bacillus subtilis* subsp. *inaquosorum* RLS52 contains some non-volatile diffusible antifungal metabolites which inhibited the mycelial growth of *Alternaria macrospora* 65.14% (Plate 2, Table 2).

Table 2 Per cent inhibition radial growth of *Alternaria macrospora* by diffusible non-volatile antifungal metabolites

Rhizospheric isolate	Radial growth of the pathogen in control plate (a control value)	Radial growth of the pathogen with antagonist (a test value)	% of inhibition *Alternaria macrospora*
Bacillus subtilis subsp. *inaquosorum* RLS52	35 mm	12.2 mm	65.14

Earlier, Arguelles-Arias *et al.* (2009) reported that *Bacillus amyloliquefaciens* GA1 produced antibiotics and other secondary metabolites such as surfactin, fengycin, iturin A, macrolactin, difficidin, Bacillaene, and Chlorotetaine for the biological control of phytopathogens. The species of *Bacillus* produces lipopeptides like the surfactin, iturin, and fengycin had suppressive mechanisms for the phytopathogens which include parasitism, competition, and antibiosis (Bonmatin *et al.* 2003). Also Ji *et al.* (2013) reported that cell free extract of *Bacillus amyloliquefaciens* CNU114001 shown antagonism against phytopathogens, *Alternaria panax*, *Botrytis cinera*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea* and *S. sclerotiorum*. Pane and Zaccardelli (2015) applied *Bacillus* sp. to control *Alternaria alternata*, the causative agent of Alternaria blight of Tomato where

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Bacillus sp. releases diffusible non-volatile metabolites. Ali *et al.* (2016) showed that cultural filtrate of B11-128 (*B. subtilis*) and B11-144 (*B. amyloliquefaciens*), possessed the strongest inhibitory activity against all three *Alternaria* sp.

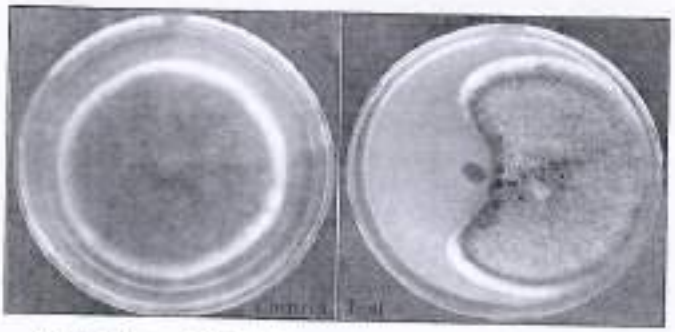

Plate 2 Effect of diffusible non-volatile antifungal metabolites produced by *Bacillus subtilis* sub sp. *inaquosorum* RLS52 against *Alternaria macrospora* in agar well diffusion method

All these reports were indicative in that the *Bacillus sp.* had ability to secret diffusible non volatile metabolites to inhibit the growth of phytopathogens. *Bacillus subtilis* subsp. *inaquosorum* RLS52 too produced diffusible non-volatile secondary metabolites which effectively inhibited the mycelia growth of *Alternaria macrospora* 65.14 percent.

Scanning electron microscopy study of co-culture

The co-culture study between, *Bacillus subtilis* subsp. *inaquosorum* RLS52 and the phytopathogen *Alternaria macrospora* in Kings B broth, revealed the lysis of mycelium and also suppression of spore formation of *Alternaria macrospora* due to the secondary metabolites produced by *Bacillus sp.* While in the control, the mycelium of the phytopathogen was in intact form and sporulation was as usual (Plate 3).

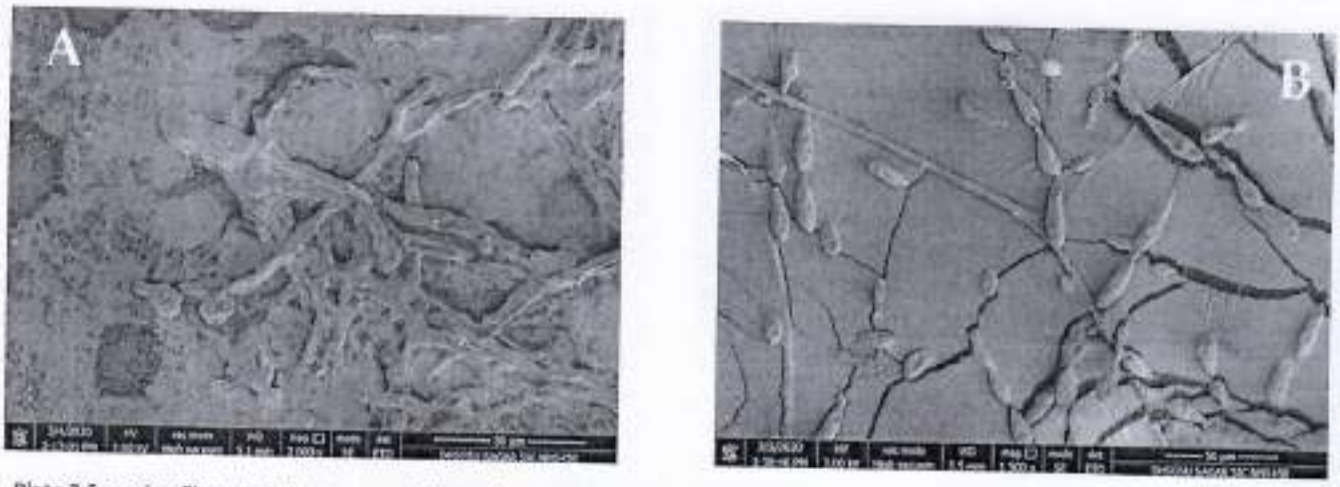

Plate 3 Scanning Electron Microscopy of effect of secondary metabolites on the growth of *Alternaria macrospora* in co-culture with *Bacillus subtilis* subsp. *inaquosorum* RLS52. (A) Lysis of mycelium and suppression of sporulation of *Alternaria macrospora* by secondary metabolites produced by *Bacillus subtilis* subsp. *inaquosorum* RLS52 (Test). (B) Intact mycelium and normal sporulation of *Alternaria macrospora* (Control)

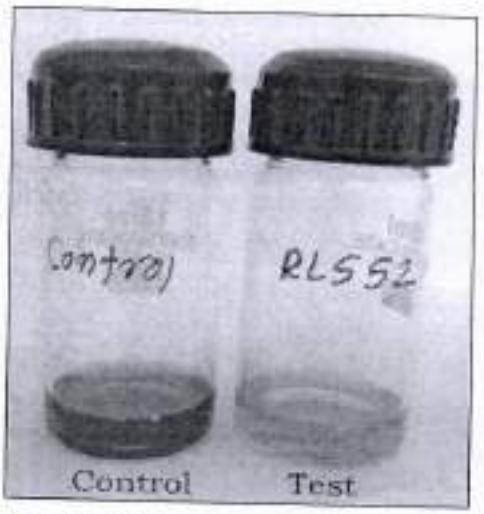

Plate 4 Detection of siderophore production quantitatively by rhizospheric isolate *Bacillus subtilis* subsp. *inaquosorum* RLS52 using liquid CAS assay

Detection of Siderophore by liquid CAS assay quantitatively

The siderophore analysis of *Bacillus subtilis* subsp. *inaquosorum* RLS52 was studied by quantitative method. The 0.5 ml cell free culture filtrate mixed with 0.5 ml of CAS solution showed the change in colour from blue to orange (Plate 4) which is indicative of siderophore production. The % siderophore units produced by efficient rhizospheric bacterial isolate in the cell free culture filtrate were calculated as per Payne, (1994). *Bacillus subtilis* subsp. *inaquosorum* RLS52 produced 85.04 % siderophore (Table 3). The time of harvesting of highest siderophore from fermentation broth was 72 h and with subsequent incubation, there was a decrease in % siderophore units and it was due to degradation of produced siderophore.

Sharma *et al.* (2013) revealed that the *B. amyloliquefaciens* strain sks_bnj_1 strain produced 82.35% siderophore units. Patil *et al.* (2014) found that *B. subtilis* CTS-G24 produced 64% and 59% siderophore units in Succinate and Nutrient media respectively. In our study,

Jaig M, Fatima S, Kadam V B and Shaikh Y. 2012. Utilization of antagonist against seed borne fungi. *Trends in Life Sciences* **1**(1): 42-46.

Bashan Y and Hernandez -Saavedra N Y. 1992. Alternaria-blight of cotton: Epidemiology and transmission. (Eds) Chelkowski J. and Visconti A. *Alternaria*: biology, plant diseases and metabolites. Elsevier Scientific Publishers, Amsterdam. pp 233-266.

Bashan Y, Levanony H and Or R. 1991. Association between *Alternaria macrospora* and *Alternaria alternata*, causal agents of cotton leaf blight. *Canadian Journal of Botany* **69**(12): 2603-2607.

Bonmatin J M, Laprévote O and Peypoux F. 2003. Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. *Combinatorial Chemistry and High Throughput Screening* **6**(6): 541-556.

Gholve V M, Jogdand S M and Suryawanshi A P. 2014. Evaluation of fungicides, botanicals and bioagents against Alternaria leaf blight caused by *Alternaria macrospora* in cotton. *Journal of Cotton Research and Development* **28**(2): 327-331.

Gveroska B and Ziberoski J. 2012. *Trichoderma harzianum* as a biocontrol agent against *Alternaria alternata* on tobacco. *ATI-Applied Technologies and Innovations* 7(2): 67-76.

Hillocks R J. 1991. Alternaria leaf spot of cotton with special reference to Zimbabwe. *International Journal of Pest Management* **37**(2): 124-128.

Hsu S C and Lockwood J L. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Environmental Microbiology* **29**(3): 422-426.

Ji S H, Gururani M A and Chun S C. 2014. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. *Microbiological Research* **169**(1): 83-98.

Kapadiya H J, Butani A M and Khanpara M D. 2015. Biological management of major foliar diseases of cotton. *Journal of Cotton Research and Development* **29**(1): 108-111.

Karunya S K. 2011. Optimization and purification of chitinase produced by *Bacillus subtilis* and its antifungal activity against plant pathogens. *International Journal of Pharmaceutical and Biological Archive* **2**(6): 1680-1685.

Maurya M K, Singh R and Tomer A. 2014. *In vitro* evaluation of antagonistic activity of *Pseudomonas fluorescens* against fungal pathogen. *Journal of Biopesticides* **7**(1): 43-46.

Meyer J A and Abdallah M A. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *Microbiology* **107**(2): 319-328.

Muthukumar A and Venkatesh A. 2013. Exploitation of fungal and endophytic bacteria for the management of leaf blight of ribbon plant. *Journal of Plant Pathology and Microbiology* **4**(10): 1-5.

Muthukumar A and Venkatesh A. 2015. Occurrence, virulence, inoculum density and plant age of *Sclerotium rolfsii Sacc.* causing collar rot of peppermint. *Journal of Plant Pathology and Microbiology* **4**(10): 1-4.

Pane C and Zaccardelli M. 2015. Evaluation of *Bacillus* strains isolated from *solanaceous* phylloplane for biocontrol of Alternaria early blight of tomato. *Biological Control* **84**: 11-18.

Patil S, Bheemaraddi M C, Shivannavar C T and Gaddad M S. 2014. Biocontrol activity of siderophore producing *Bacillus subtilis* CTS-G24 against wilt and dry root rot causing fungi in chickpea. *IOSR Journal of Agriculture and Veterinary Science* 7(9): 63-68.

Payne S M. 1994. Detection, isolation, and characterization of siderophores. *In*: *Methods in Enzymology*. Academic Press. **235**: 329-344.

Rajput R B, Solanky K U, Prajapati V P, Pawar D M and Suradkar S R. 2013. Effect of fungal and bacterial bioagents against *Alternaria alternata* (fr.) Keissler *in vitro* condition. *The Bioscan* **8**(2): 627-629.

Rane M S and Patel M K. 1956. Diseases of cotton in Bombay I. Alternaria leaf spot. *Indian Phytopathology* **9**(2): 106-113.

Reshu and Khan M M. 2012. Role of Different Microbial-origin bioactive antifungal compounds against *Alternaria spp.* causing leaf blight of mustard. *Plant Pathology Journal* **11**(1): 1-9.

Sabry S A. 1992. Microbial degradation of shrimp-shell waste. *Journal of Basic Microbiology* **32**(2): 107-111.

Schlumbaum A, Mauch F, Vogeli U and Boller T. 1986. Plant chitinases are potent inhibitors of fungal growth. *Nature* 324: 365-367.

Sharma S K, Ramesh A and Johri B N. 2013. Isolation and characterization of plant growth-promoting *Bacillus amyloliquefaciens* strain sks_bnj_1 and its influence on rhizosphere soil properties and nutrition of soybean (*Glycine max* L. Merrill). *Journal of Virology and Microbiology* **2013**: 1-19.

Skidmore A M and Dickinson C H. 1976. Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. *Transactions of the British Mycological Society* **66**(1): 57-64.

Thakaew R and Niamsup H. 2013. Inhibitory activity of *Bacillus subtilis* BCC 6327 metabolites against growth of aflatoxigenic fungi isolated from bird chili powder. *International Journal of Bioscience, Biochemistry and Bioinformatics* **3**(1): 27-32.

Tozlu E, Tekiner N A S I B E, Kotan R, and Örtücü S E R K A N. 2018. Investigation on the biological control of *Alternaria alternata*. *Indian Journal of Agricultural Sciences* **88**(8): 1241-1247.

Wang S L, Shih I L, Liang T W and Wang C H. 2002. Purification and characterization of two antifungal chitinases extracellularly produced by *Bacillus amyloliquefaciens* V656 in a shrimp and crab shell powder medium. *Journal of Agricultural and Food Chemistry* **50**(8): 2241-2248.

Whipps J M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. *New Phytologist* **107**(1): 127-142.

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Bacillus subtilis subsp. *inaquosorum* RLS52 strain produced 85.04% siderophore units after 72 hours. These results were accordance to Sharma *et al.* (2013) and found better in terms of siderophore production units.

Table 3 Detection of siderophore production quantitatively by rhizospheric isolates by using liquid CAS assay

Rhizospheric isolate	Siderophore units (%)					
	24 h	36 h	48 h	60 h	72 h	84 h
Bacillus subtilis subsp. *inaquosorum* RLS52	19.28	42.51	69.06	78.02	85.04	81.56

Detection of Chitinase enzymes

The chitinase production ability of *Bacillus subtilis* subsp. *inaquosorum* RLS52 was tested by colloidal chitin agar plate assay. The rhizobacterial isolate had shown the zone of clearance around the colony indicative of chitinase production. The zone of clearance around the colony indicates the production of chitinase enzyme and the chitinase activity measured in mm (Plate 5, Table 4).

Table 4 Detection of Chitinase enzyme production of rhizospheric isolates by colloidal chitin plate assay

Rhizospheric isolates	Total zone of hydrolysis including colony (mm)	Diameter of colony (mm)	Diameter Zone of chitin hydrolysis (mm)
Bacillus subtilis subsp. *inaquosorum* RLS52	14.7 mm	7.4 mm	7.3 mm

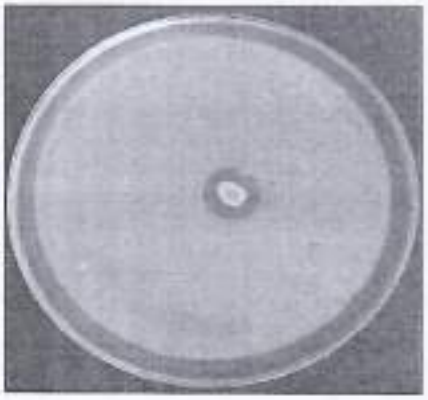

Plate 5 Chitinase enzyme activity of *Bacillus subtilis* subsp. *inaquosorum* RLS52 by colloidal chitin plate assay

The chitinase production ability of *B. amyloliquefaciens* first time recorded by researcher Sabry (1992). Wang *et al.* (2002) reported *B. amyloliquefaciens* V656 produced antifungal enzymes when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine waste. Karunya *et al.* (2011) reported that the *Bacillus subtilis* produce the enzyme chitinase and showed 0.6 mm zone of clearance around the spot inoculated culture in Colloidal Chitin agar. Thakaew and Niamsup, (2013) showed that the antagonist *Bacillus subtilis* BCC 6327 secrets protease, chitinase and β-1, 3-glucanase enzymes, which was shown to inhibit the growth and spore germination of the isolated aflatoxigenic fungus. Abirami *et al.* (2016) reported that *Bacillus licheniformis* and *Bacillus subtilis* produced prominent and maximum clear zone of 14 mm and 11 mm respectively in Colloidal Chitin Agar medium.

In the present research, *Bacillus subtilis* subsp. *inaquosorum* RLS52 produced the clear zone of 7.3 mm which was better than results obtained by Karunya *et al.* (2011), Thakaew and Niamsup (2013), Abirami *et al.* (2016). As chitin is the major constituent of cell wall of phytopathogen, the chitinase enzyme produced by rhizospheric isolate *Bacillus subtilis* subsp. *inaquosorum* RLS52 confirmed the important role played in controlling of the phytopathogens of cotton, *Alternaria macrospora.*

LITERATURE CITED

Abbo A S, Idris M O and Hammad A M. 2014. The antifungal effects of four tomato rhizosphere *Bacillus spp.* against *Alternaria alternata*. *International Journal of Science and Research* **3**(7): 1324-1328.

Abdalla S A, Algam S A, Ibrahim E A and El Naim A M. 2014. *In vitro* screening of *Bacillus* isolates for biological control of early blight disease of tomato in shambat soil. *World Journal of Agricultural Research* **2**(2): 47-50.

Abirami S, Yogalsakshmi K, Pushpa A S R and Kananan M. 2016. Screening and identification of chitin degrading bacteria from shrimp shell waste dumping soil environment and its media optimization for chitinase enzyme production. *World Journal of Pharmacy and Pharmaceutical Sciences* **5**(11): 743-757.

Akond M A, Zohora F T, Jolly S N, Mubassara S, Hossain M A and Noor R. 2016. Isolation of a potential antifungal *Bacillus subtilis* 37-JM07 strain from straw and its biocontrol efficacy to combat green mold disease of commercial mushroom, *Pleurotus ostreatus*. *Environmental Science: An Indian Journal* **12**(3): 91-103.

Ali G S, El-Sayed A S, Patel J S, Green K B, Ali M, Brennan M and Norman D. 2016. *Ex Vivo* Application of secreted metabolites produced by soil-inhabiting *Bacillus spp.* efficiently controls foliar diseases caused by *Alternaria spp. Applied and Environmental Microbiology* **82**(2): 478-490.

Arguelles-Arias A, Ongena M, Halimi B, Lara Y, Brans A, Joris B and Fickers P. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. *Microbial Cell Factories* **8**(1): 63.