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Antagonism of *Bacillus thuringiensis* NCIM2130 against *Sclerotium rolfsii Sacc.*, A Stem Rot Pathogen of Groundnut

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A B S T R A C T

Stem rot is one of the most drastic diseases of groundnut that cause major crop loss. Stem rot pathogen of groundnut, *Sclerotium rolfsii*, was isolated from the infected groundnut stem. Present investigation was started to search for effective biocontrol agent against *Sclerotium rolfsii*. To find effective biocontrol agent, 120*Bacillus spp.* isolated from various rhizospheric soils of healthy plants, screened *in vitro* against the stem rot pathogen by dual culture technique. Out of these *Bacillus spp*, *Bacillus* 57isolate found effective in controlling the phytopathogen by Novel Ring method (80.21 %). *Bacillus* 57was identified by 16S rRNA sequencing as *Bacillus thuringiensis* NCIM2130. To effectively control the phytopathogen *in vitro*, the *Bacillus thuringiensis* NCIM2130 produced the Volatile metabolites, and Siderophore.

Introduction

Modern Agriculture heavily relies on the application of chemical pesticides for disease control. Due to the concerns regarding human health and environmental issues, an alternative to these chemicals pesticides being searched (Franks *et al.*, 2006). The use of biological control approach against phytopathogens to replace hazardous effect of chemical pesticides on fertilizing soils is steadily gaining worldwide acceptance. In this regard, the use of plant growth promoting rhizobacteria (PGPR) has proven potential in developing sustainable agricultural systems for crop production and protection (Erturk *et al.*, 2010; Govindasamy *et al.*, 2011). Among the genera of bacteria, *Bacillus spp*., *Pseudomonas spp*. is widely used as biocontrol agents and *Bacillus* spp. has been reported to produce several antibiotics (Ferreira *et al.*, 1991).

Several studies reveal that *Bacillus* species are among the most prominent bacteria found to colonize plants root and soil populations (Beneduzi *et al.*, 2008). The genus *Bacillus* is characterized by Gram positive, aerobic or facultative anaerobic, rod shaped bacteria that form endospores

(Clausand Berkeley, 1986). *Bacillus* species protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as, by induction of host resistance. It largely depends on a wide variety of traits, such as the production of structurally diverse antibiotics (Liu *et al.,* 2006), production of iron chelators, bacterial phytohormones and/or the solubilization of mineral phosphates(Calvo *et al.,* 2010;Viruel *et al.,* 2011) and a ubiquitous presence in soil (Gajbhiye *et al.,* 2010). Enhancement of plant growth by root colonizing *Bacillus* sp. is well documented (Kloepper *et al.,* 2004; Idris *et al.,* 2007a; Idris *et al.,* 2007b).

In India among the soil-borne fungal diseases of groundnut, stem rot caused by *S. rolfsii* is a potential threat to production and is of considerable economic significance for groundnut grown under irrigated conditions. Stem-rot caused by *S. rolfsii* is sporadic in most of the groundnut growing areas like Tamil Nadu, Andhra Pradesh, and Karnataka (Pande and Narayana, 2000). The traditional agricultural practice to control the phytopathogen *S. rolfsii* is by using variety of fungicides e.g. Bavistin, Captan etc. but a severe disadvantage of the traditional method, that it is not effective to check the *Sclerotium* during the cropping duration (90-100 days) and is not eco-friendly. Hence, as an alternative attempt has been made to give an eco-friendly strategy for the control of *Sclerotium* during this work.

Keeping in view, the importance of rhizospheric bacteria in sustainable agriculture development by controlling the phytopathogen, the present study aims at (i) Isolation of stem rot causing pathogen of Groundnut, (ii) Screening of *Bacillus* isolates isolated from rhizospheric soil for *in vitro* antagonism against *Sclerotium rolfsii* (iii) To identify the *bacillus* isolate based on 16S rRNA sequencing and (iv) Characterization of *in vitro* biocontrol mechanism of *Bacillus isolates.*

Materials and Methods

Collection of diseased groundnut plants

Diseased groundnut stems were collected from Adgaon fields near the Purna in poly-ethylene bags and brought to laboratory, Department of Microbiology, Shri Guru Buddhiswami Mahavidyalaya, Purna (Jn.) Parbhani (Maharashtra) (Photo Plate 1).

Isolation of Phytopathogen

Diseased groundnut stem showing typical symptoms of stem rot i.e. wilting of total plants, white mycelial growth at collar region of plant (Photo Plate 1) were selected and used as source for the isolation of causative agent. Infected portion of stem was cut into small pieces with sterilized scalpel, cleaned with distilled water, then surface sterilized with 0.1% $HgCl_2$ solution for 30 second and again washed thrice with sterile distilled water. Small 1 to 2 pieces were transferred aseptically on Potato Dextrose Agar (PDA) plates containing Chloramphenicol (30 mg/100 ml) with the help of sterilized forceps under aseptic condition (Rakh, 2010). Inoculated Petri plates were incubated at 25°C for 7 days for growth of the pathogen.

Isolation of Rhizobacteria

To isolate rhizospheric *Bacillus*, soil from different healthy plants such as neem, soybean, tur and groundnut were collected in poly-ethylene bags and brought to the research laboratory. 1 gm of soil sample was inoculated into 100 ml nutrient broth and kept for incubation at room temperature for 24 h. For isolation of *Bacillus* isolates,

modified method of (Kim *et al.*, 1997) was employed. 1ml of enriched Nutrient Broth was added to 10 ml sterile distilled water and kept at 80°C for 20 min. later on a loopful of culture was streaked on nutrient agar plates and incubated at room temperature for 48 hr. After incubation, typical white colonies were picked up individually and purified on nutrient agar slants. All the isolates were tentatively named during this research to avoid confusion.

Screening for Potential Biocontrol agents

For primary screening, all the *Bacillus* isolates were screened for potential antagonistic activity against *S. rolfsii*, on King's B agar (Ran *et al.*, 2003) by using dual culture technique (Rangeshwaran and Prasad, 2000). An agar disc (5 mm) was cut from an actively growing (96 h) *S. rolfsii*, and placed on the surface of fresh King's B Agar medium at the one side of the Petri plates. A loopful of actively growing *Bacillus* isolates (each) was placed opposite to the fungal disc. Plates inoculated with phytopathogen and without bacteria were used as control. Each experiment was carried out in triplicates. Plates were incubated at room temperature for 7 days. Degree of antagonism was determined by measuring the radial growth of pathogen with bacterial culture and control and Percent inhibition was calculated by the following equation (Riungu *et al.*, 2008).

In secondary screening, efficient antagonistic *Bacillus* isolate was tested for biocontrol activity against *Sclerotium rolfsii* by Novel ring method (Adetuyiand Cartwright, 1985; Agarry *et al.*, 2005). A 5 mm diameter mycelial disc of the fungal pathogen was inoculated at the centre of a 9 cm diameter Petri plate containing king's B agar and the bacterial isolate was streaked at a distance of 2 – 3 cm from the centre in a circular pattern. The control plates were inoculated only with the fungal pathogens. The plates were incubated at 28°C and checked daily until the fungal growth on the control plate (inoculated only with *S. rolfsii*) reached the edge of the plate. Each experiment was carried out in triplicates. Percent inhibition was calculated by the following equation (Riungu *et al.*, 2008).

$$\text{Inhibition (\%)} = \frac{\text{Colony diameter of pathogen – Colony diameter of pathogen + alone (Control) Antagonist}}{\text{Colony diameter of pathogen alone}} \times 100$$

Efficient antagonistic *Bacillus* isolates were identified by using 16S rRNA sequencing. 16S rRNA sequencing of culture was carried out at Agharkar Research Institute (ARI) Pune, Maharashtra.

Characterization of Biocontrol Mechanism

To characterize the mechanism of biocontrol agent, the efficient *Bacillus* isolate tested for the production of volatile metabolite, and siderophore.

Detection of volatile metabolites

For detection of volatile antifungal metabolites produced by the *Bacillus* isolate, two half plates (sterile) were taken. The plates were poured with sterile molten and cooled King's B Agar and allowed to solidify. Now the *Bacillus* isolate was inoculated on one of the half plate in centre and on the other half the four day old pure culture of fungal phytopathogen was placed. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension. The plates were sealed to isolate the inside

atmosphere and to prevent loss of volatiles produced. Plates were incubated at room temperature for 6 days and the growth of the pathogen was measured and compared to control plates developed in the absence of the bioantagonist. Experiment was run in triplicate (Montealegro *et al.*, 2003). Results are expressed as means of inhibition (%) of the growth of fungal pathogens in the presence and absence of any bacterial isolate. Percent inhibition was calculated using the following formula (Montealegro *et al.*, 2003).

$$\text{Percent Inhibition} = \frac{100\ (C - T)}{C}$$

Where,
C = Radial growth of fungus in control plates (mm).
T = Radial growth of fungus on the plate inoculated with Antagonist (mm).

Qualitative Detection of Siderophore

Qualitative detection of siderophore was carried out by using modified CAS – blue agar(Adriane *et al.,* 1999). CAS-blue agar (1 l) was prepared according to (Schwyn and Neilands, 1997) using 60.5 mg CAS dissolved in 50 ml water distilled, deionized, and mixed with 10 ml iron (III) solution (1 mM FeCl .6H 0, 10 mM HCl). Under stirring, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml water. The resultant dark blue liquid was autoclaved at 121°C for 15 min. Also autoclaved was a mixture of 750 ml water, 15 g agar, 30.24 g Pipes, and 12 g of solution of 50 % (w/w) NaOH to raise the pH to the pKa of Pipes (6.8). The dye solution was finally poured along the glass wall and agitated with enough care to avoid foaming.

Petri dishes (10 cm in diameter) were prepared with 30 ml of king's B medium for culturing. After becoming solid, the medium was cut into halves, one of which was replaced by CAS – blue agar (15 ml). The halves containing culture medium were inoculated with strains taken from stock cultures. The inoculum was placed as far as possible from the borderline between the two media. The plates were incubated at growth temperature of each strain for seven day in the dark. Strain growth rates were daily monitored and expressed as the number of days required by the microorganism mycelia to cover the halves of Petri plates containing the culture medium. The CAS reaction rate was determined by measuring the advance of the color – change front the CAS – blue agar, starting from the borderline between the media. The CAS agar color changed from blue to orange, purple, or dark purplish – red (Adriane *et al.*, 1999).

Result and Discussion

Isolation of *Sclerotium rolfsii*

After 7 days incubation on PDA plates, the fungus produced abundant white septate mycelia, 1.5–3.0 µm diameter with clamp connections at each septation, aerial hyphae and also numerous spherical, or ellipsoidal, white sclerotia, 0.5–2.0 mm diameter, which turned brown on maturation, (Photo Plate 2). Based on morphological and culture characteristic, the disease causing organism was identified as *Sclerotium rolfsii* (Mesquita *et al.,* 2007).

Isolation of Rhizobacteria

During present research work, 120 rhizospheric *Bacillus* isolates were isolated from rhizospheric soil of different healthy plants such as Soybean, Neem, Tur etc. All the rhizospheric *Bacillus* isolates were tentatively named as *Bacillus* 1 to 120.

Screening for Biocontrol agents against Phytopathogens

The entire 120 *Bacillus* isolates were primarily screened for their antagonistic activity against isolated pathogen of groundnut viz. *S. rolfsii*, by dual culture technique which later on secondarily tested by Novel rings method. The present investigation reveals that among the 120 isolates, the *Bacillus* isolates namely *Bacillus 57* found as efficient antagonists against *S. rolfsii* during primary screening (79.35%) while comparing to other *Bacillus* isolates (Table 1). Other *Bacillus* isolates also showed considerable inhibition of *Sclerotium rolfsii in vitro* but not as efficient as *Bacillus 57*.The *Bacillus 57* secreted antifungal compound which was antagonistic and inhibited *Sclerotium rolfsii* growth 80.21 % *in vitro* in comparison to control and with absence of sclerotial production, during the secondary testing (as shown in Photo Plate 3 and Table 2). The 16 S rRNA gene sequencing of the *Bacillus 57* revealed as *Bacillus thuringiensis* NCIM2130.

Our results were also supported by other researcher's work where *Bacillus spp.* used as biocontrol agent for various disease causing pathogens affecting different plant (Killani *et al.,* 2011; Karimi *et al.,* 2012)*B. subtilis* reported to reduce the growth of S. *rolfsii* effectively on PDA when compared with the control(Helena and Ferreira,1988). This reduction in growth suggests that fungistatic material was produced by *B. subtilis* on PDA. *Bacillus subtilis*found effectively in controlling *Sclerotium rolfsii* by producing bioactive compound (Gomashe *et al.,* 2014).

In Vitro studies showed that *B. subtilis* strain, EPCO 16 showed greatest per cent inhibition (44.4) than other two strains tested on the sugarbeet root rot pathogen, *S. rolfsii* (Thilagavathi *et al.,* 2014).An inhibitory effect of *Bacillus* on wide range of fungi including *S. rolfsii* was documented by several researchers (Nalisha *et al.,* 2006; Wahyudi *et al.,* 2011). The results obtained by us was fare better than the results shown by these studies where *Sclerotium rolfsii* was inhibited *in vitro* by *Bacillus thuringiensis* NCIM2130 (80.21 %).

Characterization of Biocontrol Mechanism

To characterize the biocontrol mechanism, *Bacillus thuringiensis* NCIM2130 tested for

Detection of Volatile Metabolites

Bacillus thuringiensis NCIM2130 produced volatile metabolites *in vitro* which inhibited the growth of *Sclerotium rolfsii* upto 20 % as shown in Photo plate 4.

Table.1 Primary Screening of Bacillus isolates against *Sclerotium rolfsii* by dual culture technique

Tentative Name of Bacteria	**Inhibition of** *S. rolfsii* (%)	**Tentative Name of Bacteria**	**Inhibition of** *S. rolfsii* (%)	**Tentative Name of Bacteria**	**Inhibition of** *S. rolfsii* (%)
Bacillus 1	21.2	*Bacillus 52*	12.0	*Bacillus 103*	0.0
Bacillus 2	35.3	*Bacillus 53*	19.8	*Bacillus 104*	16.8
Bacillus 3	43.1	*Bacillus 54*	13.1	*Bacillus 105*	0.0
Bacillus 4	12.1	*Bacillus 55*	25.8	*Bacillus 106*	35.2
Bacillus 5	21.0	*Bacillus 56*	8.7	*Bacillus 107*	18.6

Bacillus 6	30.4	***Bacillus 57***	**79.35**	*Bacillus 108*	41.5
Bacillus 7	19.0	*Bacillus 58*	5.8	*Bacillus 109*	4.2
Bacillus 8	12.5	*Bacillus 59*	13.2	*Bacillus 110*	0.0
Bacillus 9	21.4	*Bacillus 60*	28.1	*Bacillus 111*	47.9
Bacillus 10	16.9	*Bacillus 61*	33.7	*Bacillus 112*	37.8
Bacillus 11	31.7	*Bacillus 62*	10.3	*Bacillus 113*	0.0
Bacillus 12	33.0	*Bacillus 63*	35.2	*Bacillus 114*	17.4
Bacillus 13	27.0	*Bacillus 64*	41.7	*Bacillus 115*	19.3
Bacillus 14	36.4	*Bacillus 65*	31.6	*Bacillus 116*	28.4
Bacillus 15	20.2	*Bacillus 66*	46.8	*Bacillus 117*	27.6
Bacillus 16	16.0	*Bacillus 67*	32.8	*Bacillus 118*	0.0
Bacillus 17	11.0	*Bacillus 68*	15.6	*Bacillus 119*	19.7
Bacillus 18	14.0	*Bacillus 69*	14.6	*Bacillus 120*	43.7
Bacillus 19	23.0	*Bacillus 70*	27.3		
Bacillus 20	26.9	*Bacillus 71*	45.8		
Bacillus 21	25.0	*Bacillus 72*	41.5		
Bacillus 22	18.7	*Bacillus 73*	37.4		
Bacillus 23	13.4	*Bacillus 74*	48.2		
Bacillus 24	15.7	*Bacillus 75*	26.4		
Bacillus 25	25.0	*Bacillus 76*	35.8		
Bacillus 26	13.8	*Bacillus 77*	11.5		
Bacillus 27	26.5	*Bacillus 78*	23.4		
Bacillus 28	34.2	*Bacillus 79*	14.7		
Bacillus 29	11.8	*Bacillus 80*	21.7		
Bacillus 30	14.3	*Bacillus 81*	29.0		
Bacillus 31	31.1	*Bacillus 82*	15.2		
Bacillus 32	25.1	*Bacillus 83*	19.4		
Bacillus 33	21.9	*Bacillus 84*	23.9		
Bacillus 34	23.2	*Bacillus 85*	31.4		
Bacillus 35	38.3	*Bacillus 86*	46.3		
Bacillus 36	27.4	*Bacillus 87*	42..6		
Bacillus 37	11.3	*Bacillus 88*	24.2		
Bacillus 38	24.8	*Bacillus 89*	33.1		
Bacillus 39	9.8	*Bacillus 90*	43.2		
Bacillus 40	17.3	*Bacillus 91*	41.2		
Bacillus 41	23.1	*Bacillus 92*	36.5		
Bacillus 42	25.3	*Bacillus 93*	39.2		
Bacillus 43	13.1	*Bacillus 94*	14.2		
Bacillus 44	29.4	*Bacillus 95*	23.6		
Bacillus 45	21.8	*Bacillus 96*	45.8		
Bacillus 46	10.9	*Bacillus 97*	34.7		
Bacillus 47	16.1	*Bacillus 98*	11.5		
Bacillus 48	20.5	*Bacillus 99*	5.3		
Bacillus 49	21.5	*Bacillus 100*	3.0		
Bacillus 50	32.1	*Bacillus 101*	0.0		
Bacillus 51	23.1	*Bacillus 102*	37.5		

Photo Plate 1: Stem Rot Disease of Ground nut

Photo Plate 2: Isolation of *Sclerotium rolfsii* **from Infected Groundnut Stem**

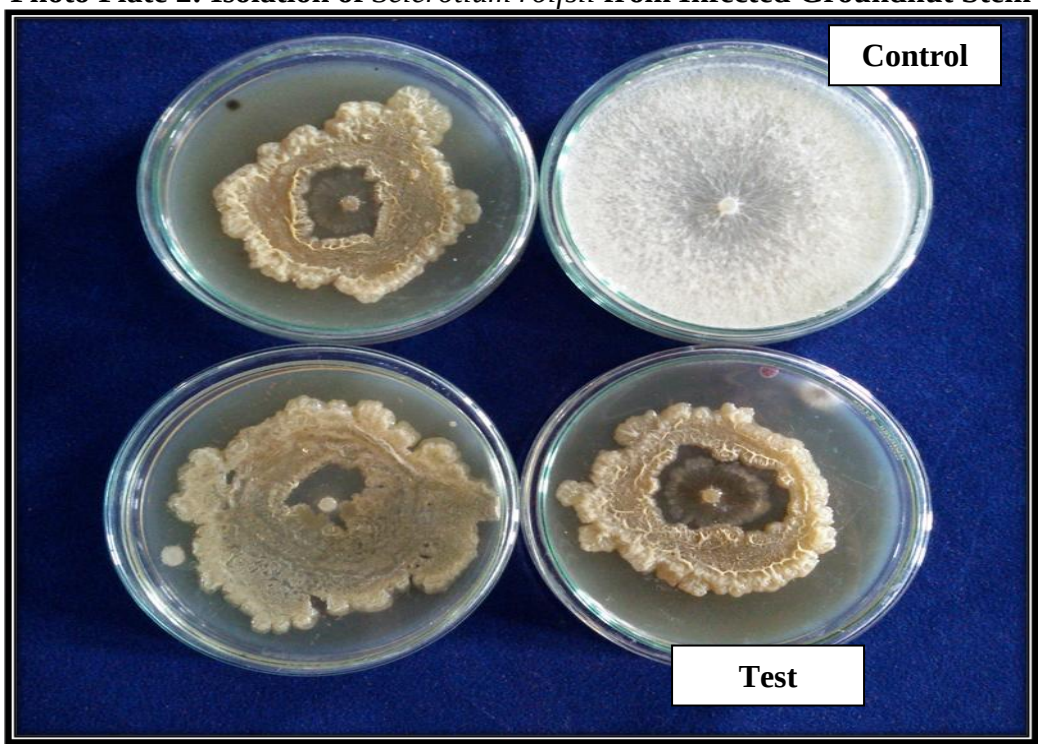

Photo Plate 3: Antagonism of *Bacillus thuringiensis* NCIM2130 **against** *Sclerotium rolfsii* **by Novel Ring method (Secondary Screening)**

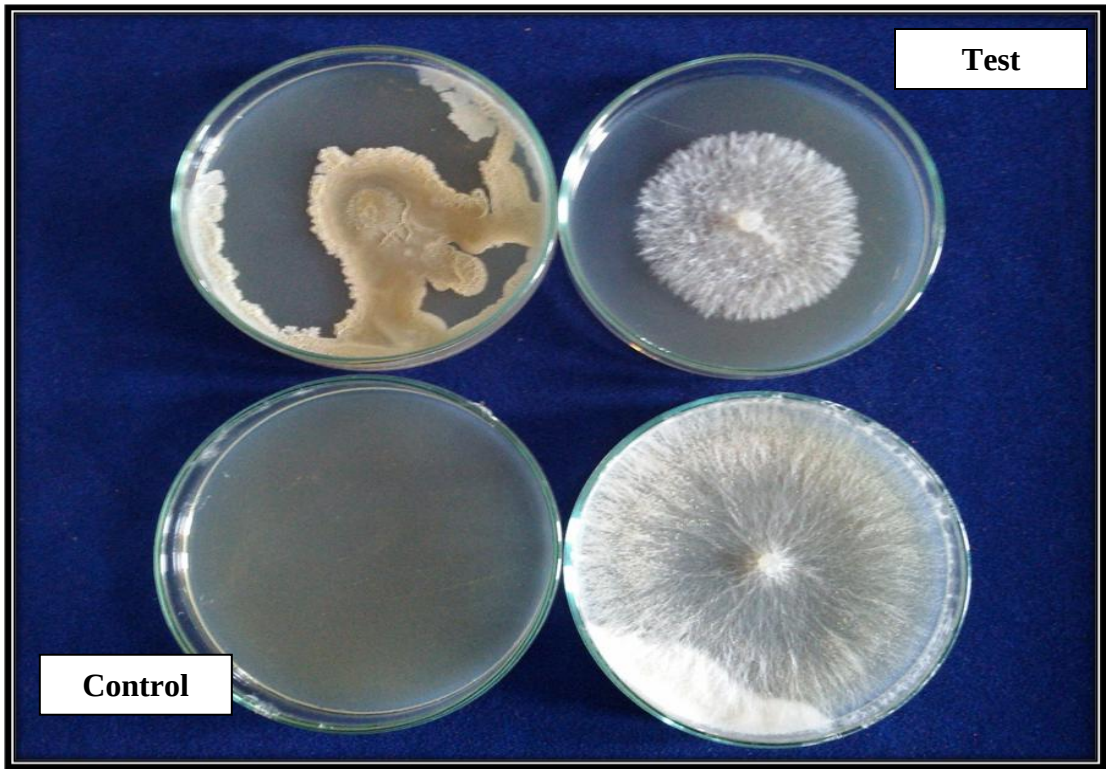

Photo Plate 4: *In Vitro* Volatile Metabolite Production by *Bacillus thuringiensis* NCIM2130

Photo plate 5: Agar plate containing CAS – blue agar and Nutrient Agar inoculated with *Bacillus thuringiensis NCIM2130*

Table.2 Secondary screening of efficient Bacillus isolate against *Sclerotium rolfsii* by Novel Ring

Bacterial culture	Test (Growth of fungal pathogen with bacterial culture in mm)	Control (Growth of fungal pathogen with out bacterial culture in mm)	Percent Inhibition%
Bacillus thuringiensis NCIM2130	24	92	**80.21**
	20	91	
	10	90	
Average	18	91	

Antibiosis mediated by volatile substances has received less attention than antibiosis through the production of non-volatile antibiotics or siderophores. Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The production of HCN by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens. *P. fluorescens CHAO* produces antibiotics, siderophores and HCN, but suppression of black rot of tobacco caused by *Thielaviopsis basicola* appeared to be due to HCN production (Voisard *et al.,* 1989). *Enterobacter cloacae* reported to produce a volatile compound such as ammonia to suppression the growth of *Pythium ultimum*-induced damping-off of cotton (Howell *et al.,* 1988). These results suggest that *Bacillus thuringiensis* NCIM2130 produced some volatile compound to inhibit *Sclerotium rolfsii.*

Qualitative Detection of Siderophore

The universal CAS assay was used form detection of siderophore production by different microorganisms in solid medium (Schwyn and Neilands, 1997) When CAS Agar plates were prepared according to Schwyn and Neilands used for detection of siderophore, most of fungi and bacteria mainly Gram positive did not grow. As stated in literature, this problem which is caused by the toxicity of HDTMA present in CAS Agar plates, affects mainly fungi and Gram positive bacteria.

When modified CAS – blue Agar plates were prepared as per (Adriane *et al.*,1999) used for detection of siderophore, *Bacillus thuringiensis* NCIM2130 grew rapidly in Nutrient agar plate, half containing medium but did not grow at all in the plate – half containing the CAS agar. The siderophore type compounds were excreted by *Bacillus thuringiensis* NCIM2130 and diffused through the CAS – blue agar, producing a colour change from blue to orange as shown in photo plate 5. For *Bacillus thuringiensis* NCIM2130 the colour change started after the *Bacillus thuringiensis* NCIM2130 had covered the plate halves containing the King's B medium for the growth (Schwyn and Neilands, 1997; Payne, 1994). This result reveals that modified CAS – blue agar found effective in qualitative detection of siderophore.

In conclusion, during this study, among 120 the *bacillus spp.* screened as biocontrol agent against *Sclerotium rolfsii*, *Bacillus thuringiensis* NCIM2130 was found effective in controlling the phytopathogen*in vitro*. This proves that the rhizospheric bacillus can be exploited as good biocontrol agent and can be tested *in vivo* in pot assay.

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