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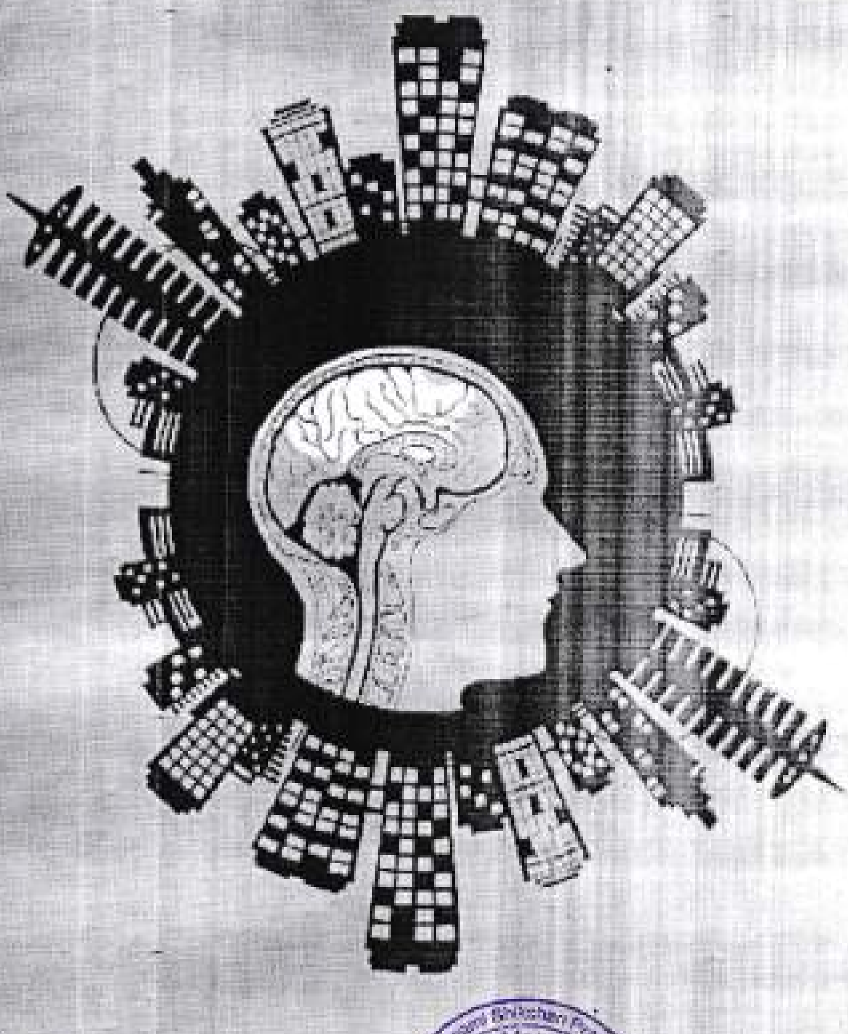

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FOR SUSTAINABLE AGRICULTURE: ISOLATION AND SCREENINGOF *BACILLUS SPP.* FOR MICROBIOLOGICAL CONTROLOF *SCLEROTIUM ROLFSII* SACC., A STEM ROT PATHOGEN OF GROUNDNUT

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Abstract:

Sclerotium rolfsii Sacc. is one of the most important pathogenof groundnutcausing Stem rot disease which causes major crop losses. In present study, to searchfor the effective Bacillus spp. for microbiological control of Sclerotium rolfsii Sacc. 189Bacillus spp. wereisolated from different rhizospheric niches of healthy plants, and primarily screened for in vitrothe antagonistic activityagainst Sclerotium rolfsii, by dual culture technique. Out of theseBacillus spp. 6, 15, 16, 18, 19, 20, 26, 29, 30, 31, 33, 34, 36, 37, 38, 39, 40, 41, 53and 57 found effectively antagonistic against Sclerotium rolfsii, the stem rot pathogen of groundnut invitro in contrast to other Bacillus spp.During the secondary screening, out of these Twenty Bacillus spp., only five Bacillus spp. i.e. Bacillus spp. 15, 16, 18, 36, and 53 found highly effective in controlling the phytopathogen, Sclerotium rolfsiiIn Vitro, in dual culture method. These Bacillus spp. 15, 16, 18, 36, and 53 effectively killing the growth of phytopathogen, Sclerotium rolfsii whose percent inhibition was 87.5, 92.30, 88.23, 80.55 and 78.37 respectively.

Key words: Groundnut, Stem rot, *Sclerotium rolfsii, Bacillus spp.*

1.0 Introduction:

Sclerotium rolfsii, a broad host range fungus, caused Stem rot, the major soilbornedisease of groundnut (*Arachis hypogaea*).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, Karnataka (Pande, *et al.*, 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 is that it is not effective to check the *Sclerotium* during the cropping period (90- 100 days) and is not eco-friendly.Becauseof the increased usage of chemical fungicidesproduced concern for the environment and humanhealth, microbial inoculants have been experimentedextensively during the last decade to control wilt andother plant diseases (Siddiqui and Shakeel, 2006;Chakraborty and Chatterjee, 2008; Akhtar *et al.*, 2010).

The use of antagonistic bacteria is reported as a powerfulstrategy to suppress soil-borne pathogens due totheir ability to antagonize the pathogen by multiplemodes and to effectively colonize the rhizosphere.The widely known mechanisms of biocontrol actionare competition for an ecological niche or substrate, aswell as the production of inhibitory compounds and hydrolyticenzymes that are often active against a broadspectrum of fungal pathogens. Many microorganismsare known to produce multiple antibiotics which cansuppress one or more pathogens (Haas and Defago,2005; Stein, 2005; Ge *et al.*, 2007). For instance, *Bacillussubtilis* produces several ribosomal and non-ribosomalpeptides that act as antibiotics such as iturins, surfactinsand zwittermycin (Asaka and Shoda, 1996; Stein, 2005)and it secretes also hydrolytic enzymes, i.e. protease,glucanase (Cazorla *et al.*, 2007), chitinase (Manjula *etal.*, 2004), lipase (Detry*et al.*, 2006) and amylase (Konsoulaand Liakopoulou-Kyriakides, 2006).

The objective of the current study was to i) isolateparticularly *Bacillus spp.*, from rhizospheric niches of healthy plants such as Neem ii) evaluate its potentialprimarilyand secondarily *in vitro*in controlling the soil-borne pathogen, *Sclerotium rolfsii*, by dual culture method.

2.0 Materials and Methods:

2.1 Chemicals:

All the chemicals used during the study were procured from M/S Hi-media, Mumbai, Glaxo Ltd., Mumbai, Sigma Aldrich, USA, unless and otherwise specified in the text. Analytical/Guaranteed (AR/GR) grade chemicals and double glass-distilled water was used.

2.2. Collection of Stem Rot Phytopathogen of Groundnut:

Sclerotium rolfsii Sacc., the Set Rot phytopathogen of groundnut used in this research work, had been isolated in previous research work conducted at Department of Microbiology, Shri Guru Buddhiswami Mahavidyalaya, Purna, Dist. Parbhani. Fungal culture of *Sclerotium rolfsii* was maintained on potato dextrose agar (PDA) by sub-culturing at regular intervals.

2.3 Isolation of Rhizospheric *Bacillus spp.*:

The present investigation was planned for isolation of an effective Microbiological control agent from soil, particularly the bacterial genera *Bacillus*, which have antagonistic potential against major groundnut diseases. Rhizospheric soil from different healthy plants such as Soybean, Neem, Jawar, Groundnut, Wheat, Tur etc. (Photo Plate 2.0) 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 spp.*, a modified method of Kim *et al.*, (1997) was employed. A 1ml of enriched nutrient broth was added to 10 ml sterile distilled water and kept at 80°C for 20 min. later a loopful of culture was streaked on nutrient agar plates. Plates were incubated at room temperature for 48 h. 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. All the isolated *Bacillus spp.* were tentatively named as *Bacillus spp.* 1 to *Bacillus spp.* 189.

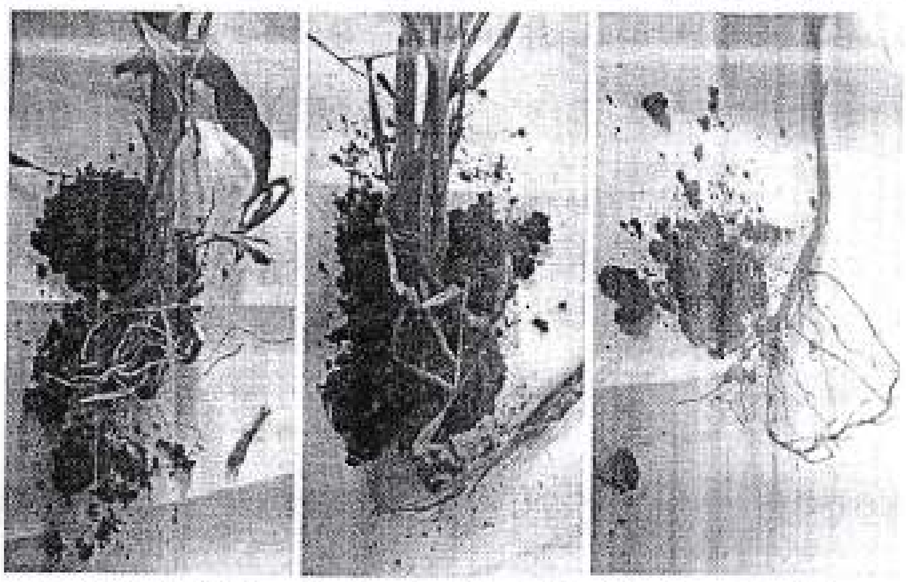

Photo Plate 2.0: Rhizospheric soil collected for isolation of *Bacillus spp.*

2.4 *In Vitro* Screening for Potential Microbiological Control Agents:

For primary screening, all the *Bacillus* isolates were screened for potential antagonistic activity against *S. rolfsii,* by using modified *dual culture technique* on King B agar plates (Gull and Hafeez, 2012, Raut and Hamde, 2016). 5 mm diameter mycelial disc was punched from margin of actively growing mycelium of *Sclerotium rolfsii* and placed at the centre of 90 mm Petri plate and *Bacillusspp.* were inoculated 30 mm apart from the centre (Figure 2.0). Three *Bacillus spp.* were placed in a plate along with phytopathogen at the centre. Control plate was kept without inoculation of rhizobacteria isolates and all the plates were incubated at room temperature for 7 days. The antifungal activity was determined by measuring the inhibition of mycelial growth of *Sclerotium rolfsii* and Percent inhibition was calculated by the following equation (Riungu *et al.*, 2008).

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

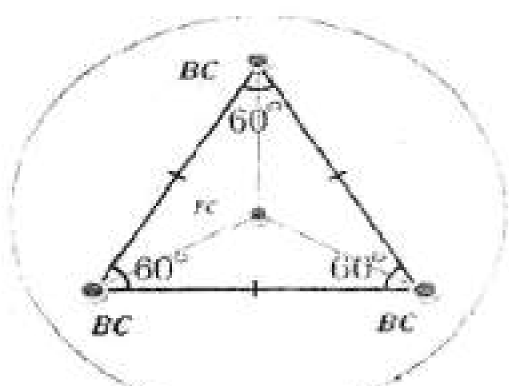

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Figure 2.0: Cardboard template for standardization dual culture technique for antifungal activity

In Secondary Screening, efficient antagonistic *Bacillus spp.* were again evaluated for microbiological control activity against *Sclerotium rolfsii* by using *dual culture technique* (Dennis and Webster, 1971) An agar disc (5 mm) was cut from an actively growing (96 h) phytopathogen, *S. rolfsii* and placed on the surface of fresh King's B agar medium at 10 mm distance from the center of Petri plate. While, the rhizobacterial *Bacillus* isolates was inoculated 10 mm away from the centre in 90 mm Petri plate containing Kings B agar. The resultant distance was 20 mm in between pathogen and antagonist in 90 mm Petri plate. Control plate was kept without inoculation of rhizobacteria isolates. 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 using the formula (Whipps, 1987)

$$\text{Percent Inhibition (\%)} = \frac{R1 - R2}{R1} \times 100$$

Where, R1 is radial growth by the pathogen in the opposite direction of the antagonist (a control value) and R2 is radial growth by the pathogen in the direction towards the antagonist (an inhibition value).

3.0 Result and Discussion:

With the recent update of Agricultural field, it has become crystal clear that groundnut is one of the most important cash crop for the farmers. Hence it is essential to improve the yield both quality and quantity wise to satisfy the demands of ever-increasing population. In this context variety of synthetic agrochemicals are used by farmers to control the Phytopathogens attacking the crop. This practice has led to many more environmental problems like: i) Disturbance of ecological balance (soil). ii) Contamination of ground water. iii) Development of resistance among the pathogens towards the synthetic chemicals. iv) Sever health risk to non target species like humans etc. To cope up with this problem an attempt has been made through this research work by using a target specific, rhizospheric bacteria for efficient control of Phytopathogens causing different disease to groundnut, in an eco-friendly and cost-effective manner.

3.1 Isolation of Rhizospheric *Bacillus spp.*:

It was well known fact that rhizospheric bacteria were excellent agents to control soil-borne plant pathogens. Rhizospheric isolates like *Bacillus*, *Pseudomonas*, *Serratia* and *Arthrobacter* have been proved to be best in controlling the fungal diseases (Handelsman and Stabb, 1996). Rhizosphere-resident antagonistic microorganisms were ideal microbiological control agents, as the rhizosphere provides the frontline defense against soil borne phytopathogens.

During present work, 189 rhizospheric *Bacillus spp.* were isolated from rhizospheric niches of different healthy plants such as Soybean, Neem, Groundnut, Tur etc. Allthe rhizospheric *Bacillus spp.* were tentatively named as *Bacillus spp* 1 to *Bacillus spp* 189 and maintained on Nutrient Agar Slants.

3.2 *In Vitro* Screening for Potential Microbiological Control Agents:

During the Primary Screening for potential microbiological control agent, the entire 189 *Bacillus spp.* were screened for their antagonistic activity against *S. rolfsii*, by *dual culture method*. The present study shown that *Bacillus spp. 6, 15, 16, 18, 19, 20, 26, 29, 30, 31, 33, 34, 36, 37, 38, 39, 40, 41, 53*and *57* recovered from the different rhizospheric niche found effectively antagonistic against *Sclerotium rolfsii*, the stem rot pathogen of groundnut *invitro* in contrast to other *Bacillus spp.* isolated from various source as shown in Photo Plate 3.0, and Table 3.0.

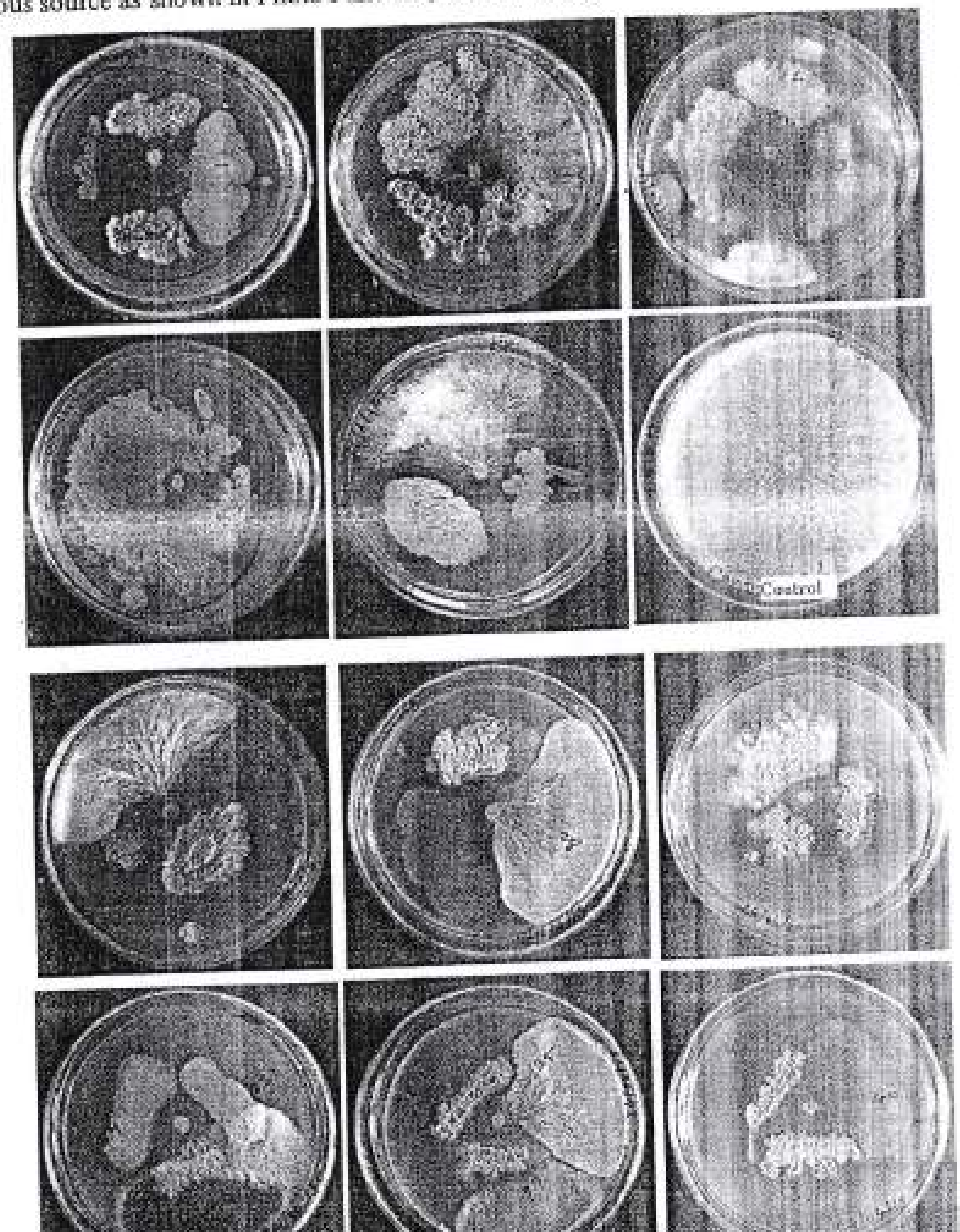

Photo Plate 3.0: ***In Vitro*** **Primary Screening of** *Bacillus spp.* against *Sclerotium rolfsii* Sacc.

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Table 3.0: *In Vitro* Primary Screening for Microbiological control Agent *Bacillus spp.* against *Sclerotium rolfsii* Sacc

Tentative Name of *Bacillus spp.*	Inhibition of *S. rolfsii* (%)	Tentative Name of *Bacillus spp.*	Inhibition of *S. rolfsii* (%)	Tentative Name of *Bacillus spp.*	Inhibition of *S. rolfsii* (%)
Bacillus spp. 1	1	*Bacillus spp. 64*	2	*Bacillus spp. 127*	0
Bacillus spp. 2	2	*Bacillus spp. 65*	2	*Bacillus spp. 128*	2
Bacillus spp. 3	2	*Bacillus spp. 66*	2	*Bacillus spp. 129*	2
Bacillus spp. 4	1	*Bacillus spp. 67*	2	*Bacillus spp. 130*	1
Bacillus spp. 5	1	*Bacillus spp. 68*	1	*Bacillus spp. 131*	1
Bacillus spp. 6	**4**	*Bacillus spp. 69*	1	*Bacillus spp. 132*	1
Bacillus spp. 7	1	*Bacillus spp. 70*	2	*Bacillus spp. 133*	2
Bacillus spp. 8	1	*Bacillus spp. 71*	2	*Bacillus spp. 134*	2
Bacillus spp. 9	1	*Bacillus spp. 72*	2	*Bacillus spp. 135*	0
Bacillus spp. 10	1	*Bacillus spp. 73*	2	*Bacillus spp. 136*	0
Bacillus spp. 11	2	*Bacillus spp. 74*	2	*Bacillus spp. 137*	1
Bacillus spp. 12	2	*Bacillus spp. 75*	2	*Bacillus spp. 138*	1
Bacillus spp. 13	2	*Bacillus spp. 76*	2	*Bacillus spp. 139*	1
Bacillus spp. 14	2	*Bacillus spp. 77*	1	*Bacillus spp. 140*	1
Bacillus spp. 15	**3**	*Bacillus spp. 78*	1	*Bacillus spp. 141*	0
Bacillus spp. 16	**4**	*Bacillus spp. 79*	1	*Bacillus spp. 142*	0
Bacillus spp. 17	1	*Bacillus spp. 80*	1	*Bacillus spp. 143*	0
Bacillus spp. 18	**4**	*Bacillus spp. 81*	2	*Bacillus spp. 144*	0
Bacillus spp. 19	**4**	*Bacillus spp. 82*	1	*Bacillus spp. 145*	0
Bacillus spp. 20	**4**	*Bacillus spp. 83*	1	*Bacillus spp. 146*	0
Bacillus spp. 21	1	*Bacillus spp. 84*	1	*Bacillus spp. 147*	0
Bacillus spp. 22	1	*Bacillus spp. 85*	2	*Bacillus spp. 148*	0
Bacillus spp. 23	1	*Bacillus spp. 86*	2	*Bacillus spp. 149*	0
Bacillus spp. 24	1	*Bacillus spp. 87*	2	*Bacillus spp. 150*	0
Bacillus spp. 25	1	*Bacillus spp. 88*	1	*Bacillus spp. 151*	0
Bacillus spp. 26	**4**	*Bacillus spp. 89*	2	*Bacillus spp. 152*	1
Bacillus spp. 27	**2**	*Bacillus spp. 90*	2	*Bacillus spp. 153*	1
Bacillus spp. 28	**2**	*Bacillus spp. 91*	2	*Bacillus spp. 154*	1
Bacillus spp. 29	**4**	*Bacillus spp. 92*	2	*Bacillus spp. 155*	1
Bacillus spp. 30	**4**	*Bacillus spp. 93*	2	*Bacillus spp. 156*	1
Bacillus spp. 31	4	*Bacillus spp. 94*	1	*Bacillus spp. 157*	1

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Bacillus spp. 32	2	*Bacillus spp. 95*	1	*Bacillus spp. 158*	2
Bacillus spp. 33	**4**	*Bacillus spp. 96*	2	*Bacillus spp. 159*	2
Bacillus spp. 34	**4**	*Bacillus spp. 97*	2	*Bacillus spp. 160*	2
Bacillus spp. 35	2	*Bacillus spp. 98*	1	*Bacillus spp. 161*	1
Bacillus spp. 36	**4**	*Bacillus spp. 99*	1	*Bacillus spp. 162*	2
Bacillus spp. 37	**4**	*Bacillus spp. 100*	1	*Bacillus spp. 163*	2
Bacillus spp. 38	**4**	*Bacillus spp. 101*	0	*Bacillus spp. 164*	1
Bacillus spp. 39	**4**	*Bacillus spp. 102*	2	*Bacillus spp. 165*	2
Bacillus spp. 40	**4**	*Bacillus spp. 103*	0	*Bacillus spp. 166*	2
Bacillus spp. 41	**4**	*Bacillus spp. 104*	1	*Bacillus spp. 167*	2
Bacillus spp. 42	2	*Bacillus spp. 105*	0	*Bacillus spp. 168*	1
Bacillus spp. 43	1	*Bacillus spp. 106*	2	*Bacillus spp. 169*	2
Bacillus spp. 44	2	*Bacillus spp. 107*	1	*Bacillus spp. 170*	0
Bacillus spp. 45	1	*Bacillus spp. 108*	2	*Bacillus spp. 171*	1
Bacillus spp. 46	1	*Bacillus spp. 109*	1	*Bacillus spp. 172*	2
Bacillus spp. 47	1	*Bacillus spp. 110*	0	*Bacillus spp. 173*	1
Bacillus spp. 48	1	*Bacillus spp. 111*	2	*Bacillus spp. 174*	2
Bacillus spp. 49	1	*Bacillus spp. 112*	2	*Bacillus spp. 175*	1
Bacillus spp. 50	2	*Bacillus spp. 113*	0	*Bacillus spp. 176*	2
Bacillus spp. 51	1	*Bacillus spp. 114*	1	*Bacillus spp. 177*	2
Bacillus spp. 52	1	*Bacillus spp. 115*	1	*Bacillus spp. 178*	0
Bacillus spp. 53	**4**	*Bacillus spp. 116*	2	*Bacillus spp. 179*	1
Bacillus spp. 54	1	*Bacillus spp. 117*	2	*Bacillus spp. 180*	1
Bacillus spp. 55	1	*Bacillus spp. 118*	0	*Bacillus spp. 181*	0
Bacillus spp. 56	1	*Bacillus spp. 119*	1	*Bacillus spp. 182*	0
Bacillus spp. 57	**4**	*Bacillus spp. 120*	2	*Bacillus spp. 183*	0
Bacillus spp. 58	1	*Bacillus spp. 121*	2	*Bacillus spp. 184*	1
Bacillus spp. 59	1	*Bacillus spp. 122*	2	*Bacillus spp. 185*	1
Bacillus spp. 60	2	*Bacillus spp. 123*	2	*Bacillus spp. 186*	1
Bacillus spp. 61	2	*Bacillus spp. 124*	0	*Bacillus spp. 187*	0
Bacillus spp. 62	1	*Bacillus spp. 125*	1	*Bacillus spp. 188*	1
Bacillus spp. 63	2	*Bacillus spp. 126*	1	*Bacillus spp. 189*	2

Each number is mean of three replicates. 0 – none, 1= inhibition zone 1 – 25 %, 2= inhibition zone 26 – 50 %, 3= inhibition zone 51 – 75 %, 4= inhibition zone 76 – 100 %.

While in Secondary Screening, all the 20 *Bacillus spp.* i.e. *6, 15, 16, 18, 19, 20, 25, 29, 30, 31, 33, 34, 36, 37, 38, 39, 40, 41, 53 and 57*, which found highly antagonistic in primary screening, were selected and screened again with *Sclerotium rolfsii by dual culture method*. Out of these Twenty

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Bacillus spp., only five *Bacillus spp*. i.e. *Bacillus spp. 15, 16, 18, 36,* and *53* found highly effective in controlling the phytopathogen, *Sclerotium rolfsiiIn Vitro*, in dual culture method (Photo Plate 3.1) These *Bacillus spp*. 15, 16, 18, 36, and 53 effectively killing the growth of phytopathogen, *Sclerotium rolfsii* whose percent inhibition was 87.5, 92.30, 88.23, 80.55 and 78.37 respectively as shown in Table 3.1

Table 3.1: ***In Vitro*** **Secondary Screening for efficient Microbiological control Agent,** ***Bacillus spp.*** **selected during primary screening against** *Sclerotium rolfsii* Sacc

Tentative Name of *Bacillus spp.*	**Radial growth by the pathogen in the opposite direction of the antagonist (a control value) R1 (mm)**	**R2 is radial growth by the pathogen in the direction towards the antagonist (an inhibition value) R2(mm)**	**Percent Inhibition (%) of *Sclerotium rolfsii***
Bacillus spp. 15	45	05	87.5
Bacillus spp. 16	39	03	92.30
Bacillus spp. 18	34	04	88.23
Bacillus spp. 36	50	06	80.55
Bacillus spp. 53	37	08	78.37

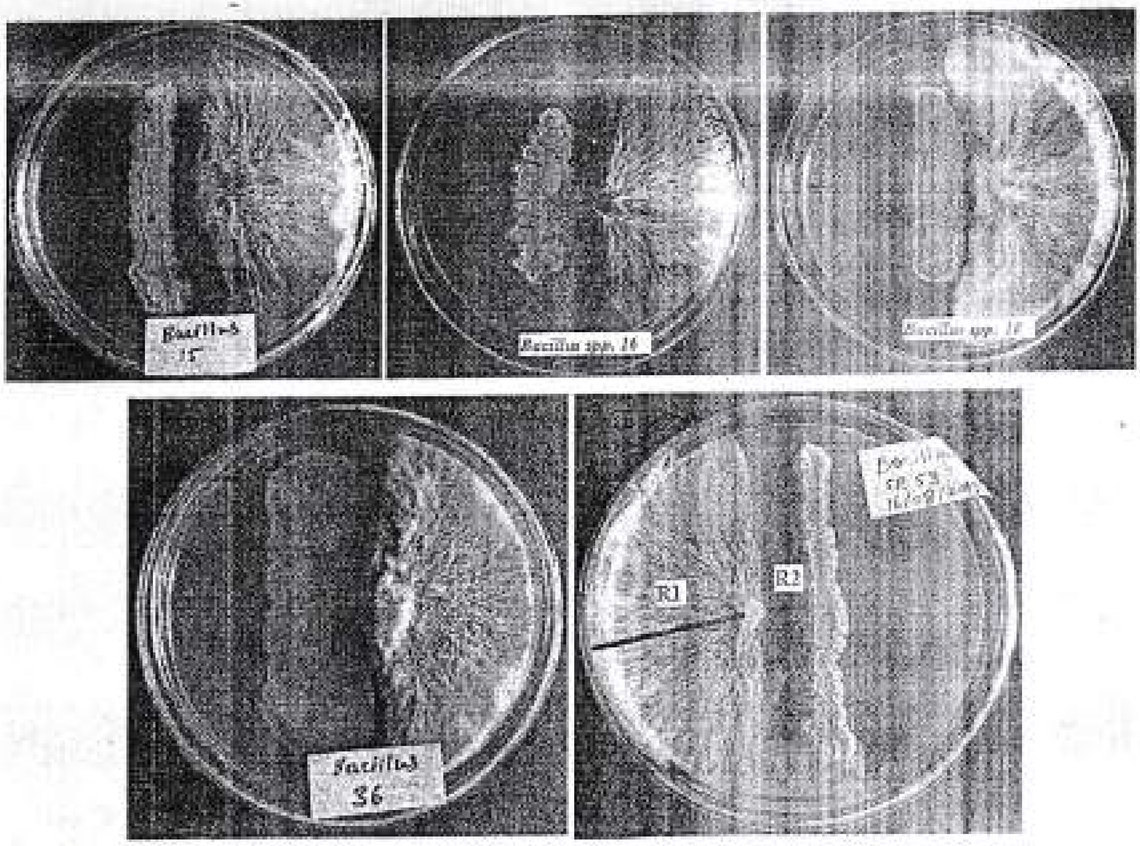

Photo Plate 3.1: - ***In Vitro*** **Secondary Screening of Efficient***Bacillus spp.***against***Sclerotium rolfsii* **in Dual Culture Method**

This result was in correlation with the result obtained by Chen *et al.* (2004). Similar findings were also recorded by the study conducted by Souto *et al.* (2004) where mycelial growth of *Sclerotium spp.* was inhibited by application of *Bacillus spp.* using the dual culture technique. Similar findings were also shown by *Bacillus subtilis* which reduced the growth of *S. rolfsii* effectively on PDA when compared with the control (Keyser and Ferreira, 1988) & also by Gomashe *et al.*, (2014) where *Bacillus subtilis* found effective in controlling *Sclerotium rolfsii* by producing bioactive compound.

Shifa*et al.*, (2015) tested a total of seven bio-control agents for their efficacy in suppressing mycelial growth of *S. rolfsii* in vitro in dual culture assay. Among the various bio-control agents tested, *B. subtilis* G-1, *B. amyloliquefaciens* B2 and *B. subtilis EPCO* 8 werefound effective in inhibiting the mycelial growth of *S. rolfsii* with mean percentage inhibition of 28, 27 and 26 respectively Similar findings were also recorded by Rajkumar *et al.*, (2018) where 30 *Bacillus subtilis* isolates were screened *in vitro* against *S. rolfsii*. The isolates showed different levels of inhibition of mycelial growth of *S. rolfsii*. Among different isolates BS16 inhibited maximum mycelial growth 64.04 per cent followed by BS 30 (11.98 %) and minimum inhibition of mycelial growth was observed in case of BS17 (11.98 %) compared to check isolate with 47 per cent inhibition of mycelial growth of *S. rolfsii.*

When all these results were compared with our results where our findings showed that *Bacillus spp.* 15, 16, 18, 36 and 53 significantly preventing mycelial growth of *Sclerotium rolfsii* in *dual culture technique* with inhibition percentage of 87.5, 92.30, 88.23, 80.55 and 78.37 respectively. Our results were far better than these results.

4.0 Acknowledgment:

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