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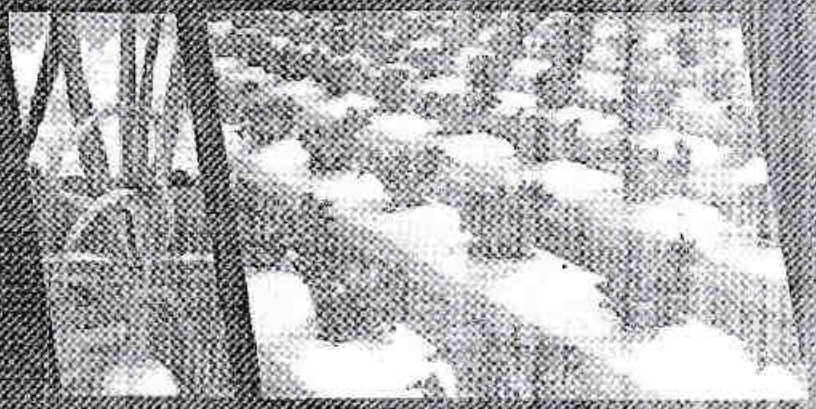
Research Journal of Agricultural Sciences

An International Journal

Volume 14 Issue 01
Jan-Feb 2023



Published by
Center for Advanced Research in Agricultural Sciences
Bhat Complex, Opp. SBI, Awantipora-192 122, Jammu & Kashmir
Contact: 01933-294741

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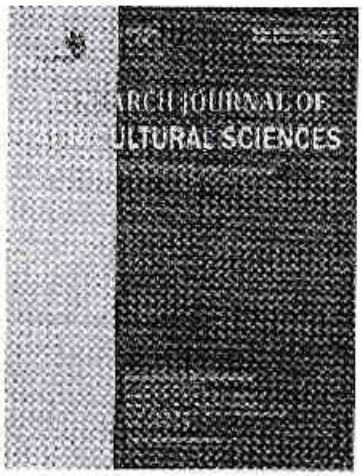

Volume - 11
May-June 2020 (Issues.aspx?IssueId=68)

Volume

Archive (Volume.aspx)

Research Journal of Agricultural Sciences: An International Journal (UGC Approved) is a bi-monthly peer reviewed research Journal, published by the Center for Advanced Research in Agricultural Sciences, devoted to the advancement and dissemination of scientific knowledge covering all disciplines related to agricultural sciences. The journal publishes original manuscripts on all aspects of agriculture and allied fields. Research papers, short communications and review articles are published based on their scientific content. All manuscripts are subjected to extensive peer reviewed by a panel of national and international referees. The journal will consider submissions from all over world on research works has not been published or is currently being considered for publication in another journal.

NAAS RATING 4.54

ISSN (Print) 0976-1675

ISSN (Online) 2249-4538

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Res. Jr. of Agril. Sci. 11(3): 499-506, May-June 2020

ISSN: 0976-1675

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Research Journal of Agricultural Sciences

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Research Paper

DI: 5956-0403-098

Bacillus spp. for *In-Vitro* Microbiological Control of *Sclerotium rolfsii* Sacc., A Stem Rot Pathogen of Groundnut

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Received: 04 March 2020; Revised accepted: 12 April 2020

ABSTRACT

Sclerotium rolfsii Sacc. is one of the most important soil borne pathogen of groundnut causing stem rot disease which causes critical crop losses in groundnut growing area. In first part of present research, stem rot pathogen of groundnut, *Sclerotium rolfsii*, was isolated from the infected groundnut plant part. In later part of research, to search for the effective *Bacillus spp.* for microbiological control of *Sclerotium rolfsii* Sacc. 189 *Bacillus spp.* were isolated from different rhizospheric niches of healthy plants, and primarily screened for *in vitro* the antagonistic activity against *Sclerotium rolfsii*, by dual culture technique. Out of these *Bacillus spp.* RRR6, RRR 15, RRR 16, RRR 18, RRR 19, RRR 20, RRR 26, RRR 29, RRR 30, RRR 31, RRR 33, RRR 34, RRR 36, RRR 37, RRR 38, RRR 39, RRR 40, RRR 41, RRR 53 and RRR 57 found effectively antagonistic against *Sclerotium rolfsii*, the stem rot pathogen of groundnut *in vitro* in contrast to other *Bacillus spp.* During the secondary screening, out of these twenty *Bacillus spp.*, only four *Bacillus spp.* i.e. *Bacillus spp.* RRR 15, RRR 16, RRR 36, and RRR 53 found extremely active in controlling the phytopathogen, *Sclerotium rolfsii In vitro* in dual culture method. These *Bacillus spp.* RRR 15, RRR 16, RRR 36, and RRR 53 effectively killing the growth of phytopathogen, *Sclerotium rolfsii* whose percent inhibition was recorded as 87.5, 92.30, 80.55 and 78.37 respectively. These *Bacillus spp.* was later identified by 16S rRNA sequencing as *Bacillus spp.* RRR15 as *Bacillus amyloliquefaciens* RRR15 (MN744706), *Bacillus spp.* RRR16 as *Bacillus amyloliquefaciens* RRR16 (MN749517), *Bacillus spp.* RRR36 as *Bacillus mojavensis* RRR36 (MN749819) and *Bacillus spp.* RRR53 as *Bacillus mojavensis* RRR53 (MN788663) respectively.

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

Sclerotium rolfsii, a broad host range fungus, caused Stem rot, the major soil borne disease 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. Because of the increased usage of chemical fungicides produced concern for the environment and human health, microbial inoculants have been experimented extensively during the last decade to control wilt and other plant diseases (Siddiqui and Shakeel 2006, Chakraborty and Chatterjee 2008, Akhtar *et al.* 2010).

Biological control is an environment-friendly strategy to reduce crop damage caused by plant pathogens. Biological control of soil-borne pathogens with antagonistic bacteria and fungi has been intensively investigated (Paulitz *et al.* 1996). Antagonistic microorganisms from rhizosphere niches are ideal biocontrol agents, as the rhizospheric niches provides the frontline defense for root against infection by

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the pathogens (Lumsden *et al.* 1995). Biocontrol of phytopathogen using antagonistic microorganism offer a highly economical and ecofriendly alternative to the use of synthetic pesticides. The use of antagonistic bacteria is reported as a powerful strategy to suppress soil-borne pathogens due to their ability to antagonize the pathogen by multiple modes and to effectively colonize the rhizosphere. The widely known mechanisms of biocontrol 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 (Haas and Defago 2005, Stein 2005, Ge *et al.* 2007). For instance, *Bacillus subtilis* produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturin, surfactin and zwittermycin (Asaka and Shoda 1996, Stein 2005) and it secretes also hydrolytic enzymes, i.e. protease, glucanase (Cazorla *et al.* 2007), chitinase (Manjula *et al.* 2004), lipase (Detry *et al.* 2006) and amylase (Konsoula and Liakopoulou-Kyriakides 2006).

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 phytopathogens, the present research aims at (i) isolate particularly Bacillus spp. RRR from rhizospheric niches of healthy plants (ii) evaluate its potential primarily and secondarily *in vitro* in controlling the soil-borne pathogen, *Sclerotium rolfsii* by dual culture method (iii) To identify the *Bacillus* isolate based on 16S rRNA sequencing.

MATERIALS AND METHODS

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.

Collection of infected groundnut plants: Infected groundnut plants (Plate 1) were collected from different locations such as, field at village Therla, Dist. Beed, from farm of Marathwada Agricultural University, Parbhani, from field at village Shirasgav, near Parbhani, and various fields from different district of Marathwada region, and brought to laboratory, Department of Microbiology, Shri Guru Buddhiswami Mahavidyalaya, Purna (Jn.) Parbhani in poly-ethylene bags.

Isolation of stem rot phytopathogen: Diseased samples showing typical symptoms of stem rot i.e. wilting of total plants, white mycelial growth at collar region of plant (Plate 1) were selected and used as sample 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 5-7 days for growth of the pathogen.

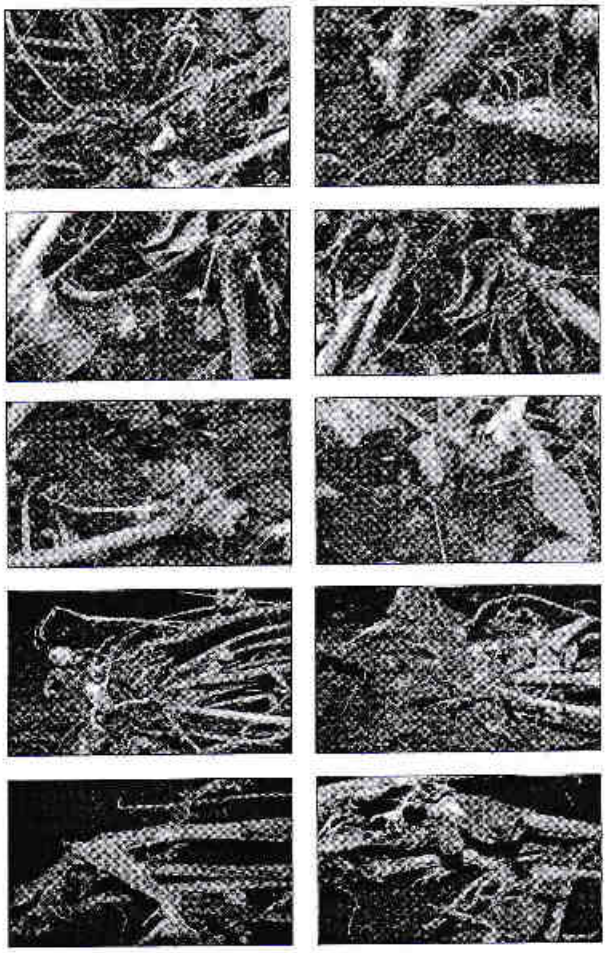

Plate 1 Stem rot infected groundnut plants

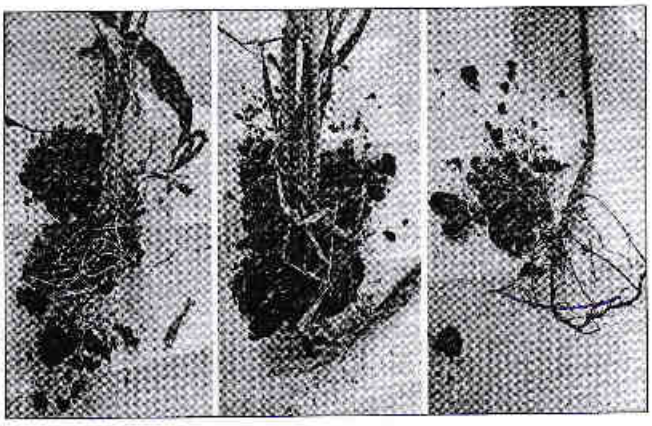

Plate 2 Soil collected for isolation of *Bacillus spp.* from rhizospheric niches of healthy plants

Isolation of Bacillus spp. from Rhizospheric Niches: The present investigation was planned for isolation of an effective Microbiological control agent from soil, particularly the bacterial genera *Bacillus*, which have

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antagonistic potential against major groundnut diseases. Rhizospheric soil from different healthy plants such as Soybean, Neem, Jawar, Groundnut, Wheat, Tur etc. (Plate 2) 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 hours.

For isolation of *Bacillus spp.* from rhizospheric niches, 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. 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.

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 *Bacillus spp.* were inoculated 30 mm apart from the centre (Fig 1). 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).

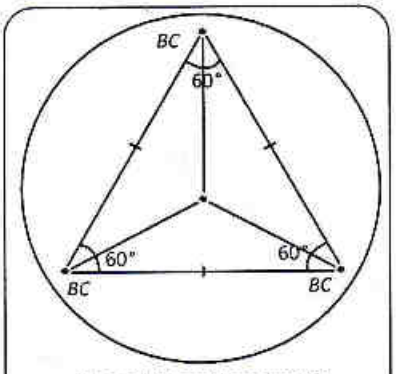

Fig 1 Cardboard template for standardization dual culture technique for antifungal activity

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

While 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 hours) 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).

Identification of effectual Bacillus spp.: The competent *Bacillus spp.* as microbiological control agent, obtained from screening was identified according to Bergey's Manual of Systematic Bacteriology (1984) by using cultural and biochemical characteristics as well as 16s rRNA sequencing. 16s rRNA sequencing of culture was carried out at Agharkar Research Institute (ARI) Pune, Maharashtra.

RESULTS AND DISCUSSION

One of the most promising alternatives to synthetic fungicides is biological control of pathogens, which includes the use of biofungicides based on antagonistic microorganisms. In contrast to commonly used fungicides, biofungicides have several advantages: high specificity against target pathogens, rapid degradation in the environment and low mass-production cost. Antagonistic microorganisms operate through various modes of activity such as competition with pathogens for space and nutrients, production of antibiotics and cell-wall degrading enzymes and reduction of pathogen population by hyperparasitism (Živković *et al.* 2010, Stanojević *et al.* 2016).

Isolation of stem rot phytopathogen

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, (Plate 3). Based on morphological and culture characteristic, the disease-causing organism was identified as *Sclerotium rolfsii* Sacc (Mesquita *et al.* 2007).

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

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phytopathogens. During present research, 189 rhizospheric *Bacillus spp.* were isolated from rhizospheric niches of different healthy plants such as Soybean, Neem, Groundnut, Tur etc. All the rhizospheric *Bacillus spp.* were tentatively named as *Bacillus spp.* RRR1 to *Bacillus spp.* RRR 189 and maintained on Nutrient Agar Slants.

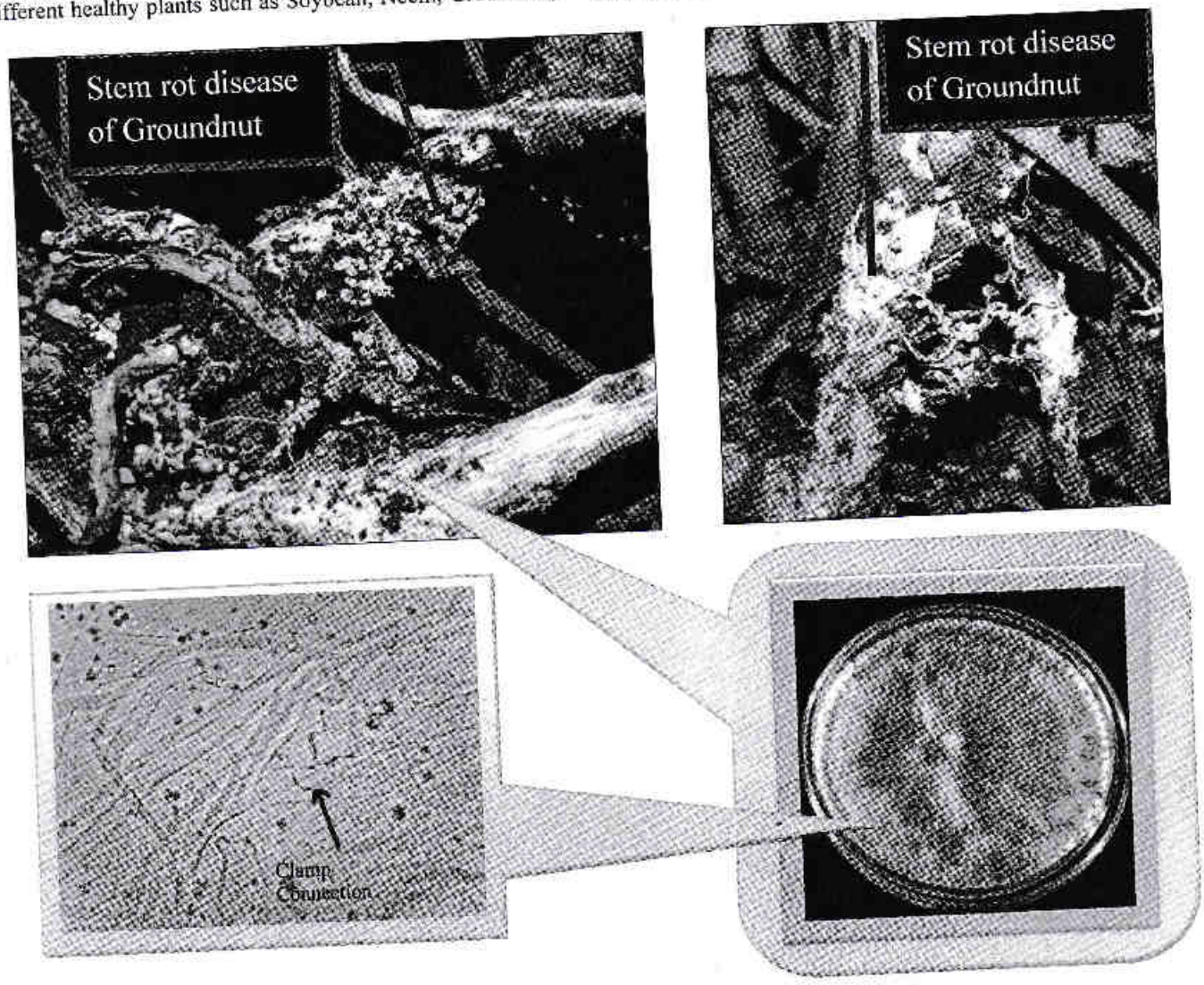

Plate 3 Isolation of stem rot phytopathogen from infected groundnut plant

Table 1 *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. RRR1	1	*Bacillus spp.* RRR 64	2	*Bacillus spp.* RRR 127	0
Bacillus spp. RRR2	2	*Bacillus spp.* RRR 65	2	*Bacillus spp.* RRR 128	2
Bacillus spp. RRR 3	2	*Bacillus spp.* RRR 66	2	*Bacillus spp.* RRR 129	2
Bacillus spp. RRR 4	1	*Bacillus spp.* RRR 67	2	*Bacillus spp.* RRR 130	1
Bacillus spp. RRR 5	1	*Bacillus spp.* RRR 68	1	*Bacillus spp.* RRR 131	1
Bacillus spp. RRR 6	4	*Bacillus spp.* RRR 69	1	*Bacillus spp.* RRR 132	1
Bacillus spp. RRR 7	1	*Bacillus spp.* RRR 70	2	*Bacillus spp.* RRR 133	2
Bacillus spp. RRR 8	1	*Bacillus spp.* RRR 71	2	*Bacillus spp.* RRR 134	2
Bacillus spp. RRR 9	1	*Bacillus spp. RRR* 72	2	*Bacillus spp.* RRR 135	0
Bacillus spp. RRR 10	1	*Bacillus spp.* RRR 73	2	*Bacillus spp.* RRR 136	0
Bacillus spp. RRR 11	2	*Bacillus spp.* RRR 74	2	*Bacillus spp.* RRR 137	1
Bacillus spp. RRR 12	2	*Bacillus spp.* RRR 75	2	*Bacillus spp.* RRR 138	1
Bacillus spp. RRR 13	2	*Bacillus spp.* RRR 76	2	*Bacillus spp.* RRR 139	1
Bacillus spp. RRR 14	2	*Bacillus spp.* RRR 77	1	*Bacillus spp.* RRR 140	1

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Bacillus spp. RRR 15	3	*Bacillus spp.* RRR 78	1	*Bacillus spp.* RRR 141	0
Bacillus spp. RRR 16	4	*Bacillus spp.* RRR 79	1	*Bacillus spp.* RRR 142	0
Bacillus spp. RRR 17	1	*Bacillus spp.* RRR 80	1	*Bacillus spp.* RRR 143	0
Bacillus spp. RRR 18	4	*Bacillus spp.* RRR 81	2	*Bacillus spp.* RRR 144	0
Bacillus spp. RRR 19	4	*Bacillus spp.* RRR 82	1	*Bacillus spp.* RRR 145	0
Bacillus spp. RRR 20	4	*Bacillus spp.* RRR 83	1	*Bacillus spp.* RRR 146	0
Bacillus spp. RRR 21	1	*Bacillus spp.* RRR 84	1	*Bacillus spp.* RRR 147	0
Bacillus spp. RRR 22	1	*Bacillus spp.* RRR 85	2	*Bacillus spp.* RRR 148	0
Bacillus spp. RRR 23	1	*Bacillus spp.* RRR 86	2	*Bacillus spp.* RRR 149	0
Bacillus spp. RRR 24	1	*Bacillus spp.* RRR 87	2	*Bacillus spp.* RRR 150	0
Bacillus spp. RRR 25	1	*Bacillus spp.* RRR 88	1	*Bacillus spp.* RRR 151	0
Bacillus spp. RRR 26	4	*Bacillus spp.* RRR 89	2	*Bacillus spp.* RRR 152	1
Bacillus spp. RRR 27	2	*Bacillus spp.* RRR 90	2	*Bacillus spp.* RRR 153	1
Bacillus spp. RRR 28	2	*Bacillus spp.* RRR 91	2	*Bacillus spp.* RRR 154	1
Bacillus spp. RRR 29	4	*Bacillus spp.* RRR 92	2	*Bacillus spp.* RRR 155	1
Bacillus spp. RRR 30	4	*Bacillus spp.* RRR 93	2	*Bacillus spp.* RRR 156	1
Bacillus spp. RRR 31	4	*Bacillus spp.* RRR 94	1	*Bacillus spp.* RRR 157	1
Bacillus spp. RRR 32	2	*Bacillus spp.* RRR 95	1	*Bacillus spp.* RRR 158	2
Bacillus spp. RRR 33	4	*Bacillus spp.* RRR 96	2	*Bacillus spp.* RRR 159	2
Bacillus spp. RRR 34	4	*Bacillus spp.* RRR 97	2	*Bacillus spp.* RRR 160	2
Bacillus spp. RRR 35	2	*Bacillus spp.* RRR 98	1	*Bacillus spp.* RRR 161	1
Bacillus spp. RRR 36	4	*Bacillus spp.* RRR 99	1	*Bacillus spp.* RRR 162	2
Bacillus spp. RRR 37	4	*Bacillus spp.* RRR 100	1	*Bacillus spp.* RRR 163	2
Bacillus spp. RRR 38	4	*Bacillus spp.* RRR 101	0	*Bacillus spp.* RRR 164	1
Bacillus spp. RRR 39	4	*Bacillus spp.* RRR 102	2	*Bacillus spp.* RRR 165	2
Bacillus spp. RRR 40	4	*Bacillus spp.* RRR 103	0	*Bacillus spp.* RRR 166	2
Bacillus spp. RRR 41	4	*Bacillus spp.* RRR 104	1	*Bacillus spp.* RRR 167	2
Bacillus spp. RRR 42	2	*Bacillus spp.* RRR 105	0	*Bacillus spp.* RRR 168	1
Bacillus spp. RRR 43	1	*Bacillus spp.* RRR 106	2	*Bacillus spp.* RRR 169	2
Bacillus spp. RRR 44	2	*Bacillus spp.* RRR 107	1	*Bacillus spp.* RRR 170	0
Bacillus spp. RRR 45	1	*Bacillus spp.* RRR 108	2	*Bacillus spp.* RRR 171	1
Bacillus spp. RRR 46	1	*Bacillus spp.* RRR 109	1	*Bacillus spp.* RRR 172	2
Bacillus spp. RRR 47	1	*Bacillus spp.* RRR 110	0	*Bacillus spp.* RRR 173	1
Bacillus spp. RRR 48	1	*Bacillus spp.* RRR 111	2	*Bacillus spp.* RRR 174	2
Bacillus spp. RRR 49	1	*Bacillus spp.* RRR 112	2	*Bacillus spp.* RRR 175	1
Bacillus spp. RRR 50	2	*Bacillus spp.* RRR 113	0	*Bacillus spp.* RRR 176	2
Bacillus spp. RRR 51	1	*Bacillus spp.* RRR 114	1	*Bacillus spp.* RRR 177	2
Bacillus spp. RRR 52	1	*Bacillus spp.* RRR 115	1	*Bacillus spp.* RRR 178	0
Bacillus spp. RRR 53	4	*Bacillus spp.* RRR 116	2	*Bacillus spp.* RRR 179	1
Bacillus spp. RRR 54	1	*Bacillus spp.* RRR 117	2	*Bacillus spp.* RRR 180	1
Bacillus spp. RRR 55	1	*Bacillus spp.* RRR 118	0	*Bacillus spp.* RRR 181	0
Bacillus spp. RRR 56	1	*Bacillus spp.* RRR 119	1	*Bacillus spp.* RRR 182	0
Bacillus spp. RRR 57	4	*Bacillus spp.* RRR 120	2	*Bacillus spp.* RRR 183	0
Bacillus spp. RRR 58	1	*Bacillus spp.* RRR 121	2	*Bacillus spp.* RRR 184	1
Bacillus spp. RRR 59	1	*Bacillus spp.* RRR 122	2	*Bacillus spp.* RRR 185	1
Bacillus spp. RRR 60	2	*Bacillus spp.* RRR 123	2	*Bacillus spp.* RRR 186	1
Bacillus spp. RRR 61	2	*Bacillus spp.* RRR 124	0	*Bacillus spp.* RRR 187	0
Bacillus spp. RRR 62	1	*Bacillus spp.* RRR 125	1	*Bacillus spp.* RRR 188	1
Bacillus spp. RRR 63	2	*Bacillus spp.* RRR 126	1	*Bacillus spp.* RRR 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%

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.* RRR 6, RRR 15, RRR 16, RRR 18, RRR 19, RRR 20, RRR 26, RRR 29, RRR 30, RRR 31, RRR 33, RRR 34, RRR 36, RRR 37, RRR 38, RRR 39, RRR 40, RRR 41, RRR 53 and RRR 57 recovered from the different rhizospheric niche found effectively antagonistic against *Sclerotium rolfsii*, the stem rot pathogen of groundnut *in vitro* in contrast to other *Bacillus spp.* isolated from various source (Plate 4, Table 1).

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While in Secondary Screening, all the 20 *Bacillus spp.* i.e. RRR 6, RRR 15, RRR 16, RRR 18, RRR 19, RRR 20, RRR 26, RRR 29, RRR 30, RRR 31, RRR 33, RRR 34, RRR 36, RRR 37, RRR 38, RRR 39, RRR 40, RRR 41, RRR 53 and RRR 57, which found highly antagonistic in primary screening, were selected and screened again with *Sclerotium rolfsii* by *dual culture method*. Out of these Twenty *Bacillus spp.*, only four *Bacillus spp.* i.e. Bacillus spp. RRR15, RRR16, RRR36, and RRR53 found highly effective in controlling the phytopathogen, *Sclerotium rolfsii In Vitro*, in dual culture method (Plate 5) These *Bacillus spp.* RRR 15, 16, 36, and 53 effectively killing the growth of phytopathogen, *Sclerotium rolfsii* whose percent inhibition was 87.5, 92.30, 80.55 and 78.37 respectively (Table 2).

Table 2 *In-vitro* secondary screening for effectual 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. RRR 15	45	05	87.5
Bacillus spp. RRR 16	39	03	92.30
Bacillus spp. RRR 36	50	06	80.55
Bacillus spp. RRR 53	37	08	78.37

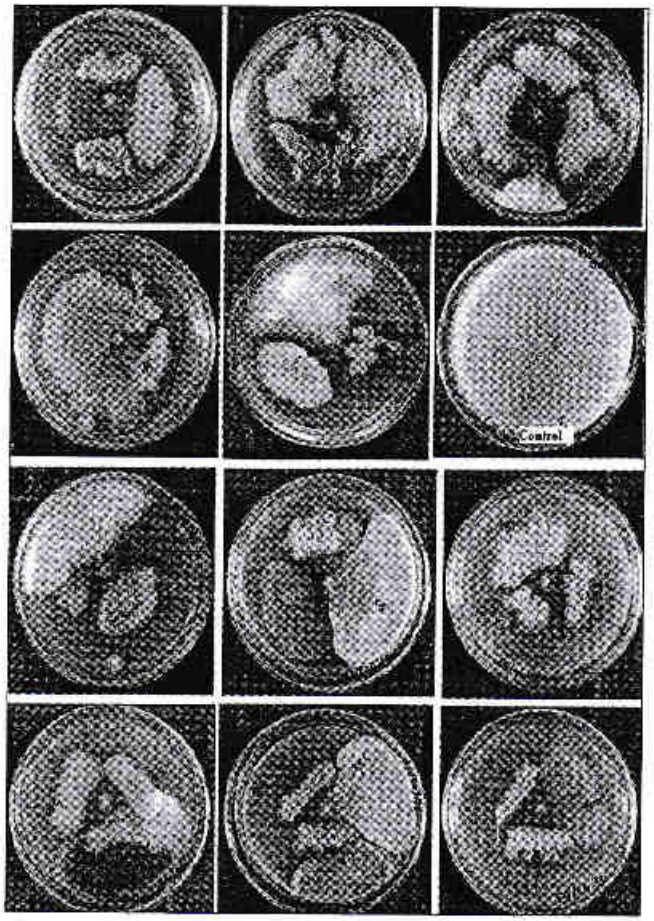

Plate 4 *In-vitro* primary screening of *Bacillus spp.* against *Sclerotium rolfsii* Sacc.

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. RRR 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) and also by Gomashe *et al.* (2014) where *Bacillus subtilis* found effective in controlling *Sclerotium rolfsii* by producing bioactive compound.

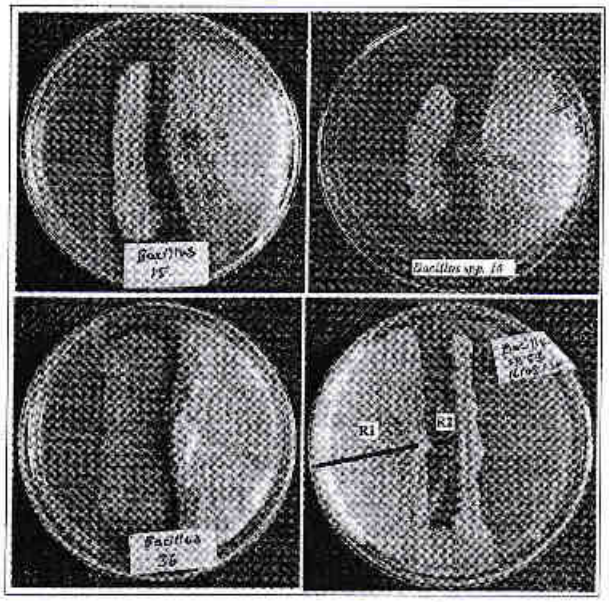

Plate 5 *In-vitro* secondary screening of efficient *Bacillus spp.* against *Sclerotium rolfsii* in dual culture method

Seven bio-control agents were tested by Shifa *et al.*, (2015) 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 were found 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 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.* RRR15, RRR16, RRR36 and RRR53 significantly preventing mycelial growth of *Sclerotium rolfsii* in dual culture technique with inhibition percentage of 87.5, 92.30, 80.55 and 78.37 respectively. Our findings were far better than these previously recorded results by Keyser and Ferreira (1988), Gomashe *et al.* (2014), Shifa *et al.* (2015), Rajkumar *et al.* (2018).

Most bacterial strains commercially used as biofungicides belong to the genera *Bacillus* and *Pseudomonas* (Fravel 2005). *Bacillus spp.* produces spores that are resistant to various physical and chemical treatments such as heat, desiccation, UV irradiation and organic solvents (Leelasuphakul *et al.* 2008). Also, they are known to produce an array of secondary metabolites, including antibiotics, cell-wall degrading enzymes and antifungal volatile substances. This indicates that Bacillus spp. strains can be efficient biological control agents against a wide range of plant pathogens (Kim and Chung 2004, Leelasuphakul *et al.* 2006). Many microorganisms are known to produce multiple antibiotics which can suppress one or more pathogens (Haas and Defago 2005, Stein 2005, Ge *et al.* 2007). For instance, Bacillus subtilis produces several ribosomal and non-ribosomal peptides that act as antibiotics such as iturin, surfactin and zwittermycin (Asaka and Shoda 1996, Stein 2005) and it secretes also hydrolytic enzymes, i.e. protease, glucanase (Cazorla *et al.* 2007), chitinase (Manjula *et al.* 2004), lipase (Detry *et al.* 2006) and amylase (Konsoula and Liakopoulou-Kyriakides 2006).

Identification of Bacillus spp.

16S rRNA sequencing and Phylogenetic analysis identified *Bacillus spp.* RRR15 as *Bacillus amyloliquefaciens* RRR15 (MN744706), *Bacillus spp.* RRR16 as *Bacillus amyloliquefaciens* RRR16 (MN749517), *Bacillus spp.* RRR36 as *Bacillus mojavensis* RRR36 (MN749819) and *Bacillus spp.* RRR53 as *Bacillus mojavensis* RRR53 (MN788663) respectively. The 16S rRNA sequence has been deposited in Genbank of National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine 8600 Rockville Pike, Bethesda MD, 20894 USA with their accession No.

Acknowledgement

The authors were grateful for the financial support provided by Swami Ramanand Teerth Marathwada University, Nanded, under Rajiv Gandhi Science and Technology Commission, (RGSTC) [Government of Maharashtra] Project to Dr. R. R. Rakh, Department of Microbiology, Shri Guru Buddhiswami Mahavidyalaya, Purna (Jn.).

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