Think India Journal
ISSN: 0971-1260 Vol-22, Special Issue-31
National Conference ETDAB-2019
Held on 23th & 24th December 2019
Organized by: Deptt. of Botany, Deogiri College, Aurangabad, M.S

In Vitro Dominance of *Bacillus amyloliquefaciens*RRR15for Microbiological Controlof *Sclerotium rolfsii* Sacc., AStem Rot Pathogen of Groundnut

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Abstract:

Stem rot disease caused by *Sclerotium rolfsii* Sacc. is one of the most important disease of groundnut causing major crop losses. To pursuit for the effective *Bacillus spp.*as microbiological control of *Sclerotium rolfsii* Sacc., 189*Bacillus spp.* were isolated from different rhizospheric nichesof healthy plants, and primarily screened*in vitro*for the antagonistic activity against *Sclerotium rolfsii,* by dual culture technique. During the primary screening, *Bacillus spp.* RRR15,found highly effective in killing the phytopathogen, *Sclerotium rolfsii in Vitro*. The*Bacillus spp.*RRR15, effectively slaughter the growth of phytopathogen, *Sclerotium rolfsii* whose percent inhibition recorded as 87.5. The *Bacillus spp.* RRR15 later identified as *Bacillus amyloliquefaciens* RRR15 by 16 S rRNA sequencing.

Key words: Groundnut, Stem rot, *Sclerotium rolfsii*, *Bacillus amyloliquefaciens* RRR15.

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 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 fungicides, produced 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).

One of the most promising alternatives to syntheticfungicides is biological control of pathogens, whichincludes the use of biofungicides based on antagonisticmicroorganisms.In contrast to commonly used fungicides,biofungicides have several advantages: highspecificity against target pathogens, rapid degradationin the environment and low mass-production cost.Antagonistic microorganisms operate through variousmodes of activity such as competition with pathogensfor space and nutrients, production of antibiotics andcell-wall degrading enzymes and reduction of pathogenpopulation by hyperparasitism (Živković*et al.*, 2010,Stanojević *et al.*, 2016).

Most bacterial strains commercially used as biofungicidesbelong to the genera *Bacillus* and *Pseudomonas* (Fravel, 2005). *Bacillus* spp. produces spores that areresistant to various physical and chemical treatmentssuch as heat, desiccation, UV irradiation and organicsolvents (Leelasuphakul*et al.*, 2008). Also, they are known to produce an arrayof secondary metabolites, including antibiotics,cell-wall degrading enzymes and antifungal volatilesubstances. This indicates that *Bacillus* spp. strains canbe efficient biological control agents against a widerange of plant pathogens (Kim and Chung, 2004;Leelasuphakul*et al.*,2006). 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).

Hence, as an alternative attempt has been madeto give an eco-friendly strategy for the control of*Sclerotium* during this work. Keeping in view,the importance of rhizospheric bacteria insustainable agriculture development bycontrolling the phytopathogens, the present studyaims at (i) isolate particularly *Bacillus spp.*from rhizospheric niches of healthy plants such as Neem, (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 16SrRNA sequencing.

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 Stem 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. 1gm 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.

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Photo plate 2.0: Soil collected from Rhizospheric niches of healthy plants 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 (Whipps, 1987,Raut and Hamde, 2016).

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

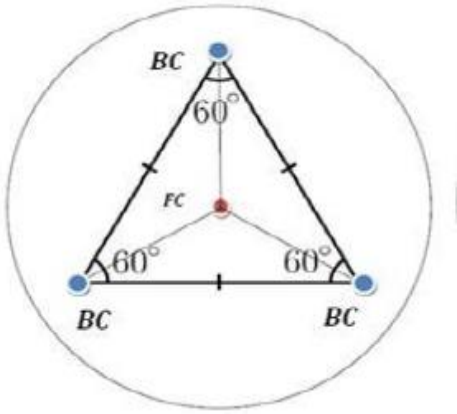


Figure 2.0: Cardboard template for standardization dual culture technique for antifungal activity

In secondary screening, efficient antagonistic *Bacillus spp.* were again evaluated for microbiological control activity against *Sclerotium rolfsii* by usingdual 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 the 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 isolate. 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, Raut and Hamde, 2016).

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

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).

2.5 Identification of Efficient ***Bacillus spp.*****:**

The efficient *Bacillus spp.*as microbiological control agent, obtainedfrom screening was identified according to Bergey's Manual ofSystematic Bacteriology (1984) by using cultural andbiochemicalcharacteristics as well as 16s rRNA sequencing. 16s rRNA sequencingof culture was carried out at Agharkar Research Institute (ARI) Pune,Maharashtra.

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, animals, birds as well as beneficial soil microflora. 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 microbial control 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, the typical white colonies were picked up individually and transferred on nutrient agar slants.Total 189 rhizospheric *Bacillus spp.* were isolated from rhizospheric niches of different healthy plants. Allthe rhizospheric *Bacillus spp.* were tentatively named as RRR1 to RRR189 during this research to avoid confusion and maintained on Nutrient agarslants.

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.*RRR6, RRR15, RRR16, RRR18, RRR19, RRR20, RRR26, RRR29, RRR30, RRR31, RRR33, RRR34, RRR36, RRR37, RRR38, RRR39, RRR40, RRR41, RRR53 and RRR57recovered 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 Table 3.0.

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

Isolates of *Bacillus spp.*	**Inhibition levels**	**Isolates of** *Bacillus spp.*	**Inhibition levels**	**Isolates of** *Bacillus spp.*	**Inhibition levels**
RRR 1	1	RRR 64	2	RRR 127	0
RRR 2	2	RRR 65	2	RRR 128	2
RRR 3	2	RRR 66	2	RRR 129	2
RRR 4	1	RRR 67	2	RRR 130	1
RRR 5	1	RRR 68	1	RRR 131	1
RRR 6	3	RRR 69	1	RRR 132	1
RRR 7	1	RRR 70	2	RRR 133	2
RRR 8	1	RRR 71	2	RRR 134	2
RRR 9	1	RRR 72	2	RRR 135	0
RRR 10	1	RRR 73	2	RRR 136	0
RRR 11	2	RRR 74	2	RRR 137	1
RRR 12	2	RRR 75	2	RRR 138	1
RRR 13	2	RRR 76	2	RRR 139	1
RRR 14	2	RRR 77	1	RRR 140	1
RRR 15	**4**	RRR 78	1	RRR 141	0
RRR 16	3	RRR 79	1	RRR 142	0
RRR 17	1	RRR 80	1	RRR 143	0
RRR 18	3	RRR 81	2	RRR 144	0
RRR 19	3	RRR 82	1	RRR 145	0
RRR 20	3	RRR 83	1	RRR 146	0
RRR 21	1	RRR 84	1	RRR 147	0
RRR 22	1	RRR 85	2	RRR 148	0
RRR 23	1	RRR 86	2	RRR 149	0
RRR 24	1	RRR 87	2	RRR 150	0

RRR 25	1	RRR 88	1	RRR 151	0
RRR 26	3	RRR 89	2	RRR 152	1
RRR 27	2	RRR 90	2	RRR 153	1
RRR 28	2	RRR 91	2	RRR 154	1
RRR 29	3	RRR 92	2	RRR 155	1
RRR 30	3	RRR 93	2	RRR 156	1
RRR 31	3	RRR 94	1	RRR 157	1
RRR 32	2	RRR 95	1	RRR 158	2
RRR 33	3	RRR 96	2	RRR 159	2
RRR 34	3	RRR 97	2	RRR 160	2
RRR 35	2	RRR 98	1	RRR 161	1
RRR 36	3	RRR 99	1	RRR 162	2
RRR 37	3	RRR 100	1	RRR 163	2
RRR 38	3	RRR 101	0	RRR 164	1
RRR 39	3	RRR 102	2	RRR 165	2
RRR 40	3	RRR 103	0	RRR 166	2
RRR 41	3	RRR 104	1	RRR 167	2
RRR 42	2	RRR 105	0	RRR 168	1
RRR 43	1	RRR 106	2	RRR 169	2
RRR 44	2	RRR 107	1	RRR 170	0
RRR 45	1	RRR 108	2	RRR 171	1
RRR 46	1	RRR 109	1	RRR 172	2
RRR 47	1	RRR 110	0	RRR 173	1
RRR 48	1	RRR 111	2	RRR 174	2
RRR 49	1	RRR 112	2	RRR 175	1
RRR 50	2	RRR 113	0	RRR 176	2
RRR 51	1	RRR 114	1	RRR 177	2
RRR 52	1	RRR 115	1	RRR 178	0
RRR 53	3	RRR 116	2	RRR 179	1
RRR 54	1	RRR 117	2	RRR 180	1
RRR 55	1	RRR 118	0	RRR 181	0
RRR 56	1	RRR 119	1	RRR 182	0
RRR 57	3	RRR 120	2	RRR 183	0
RRR 58	1	RRR 121	2	RRR 184	1
RRR 59	1	RRR 122	2	RRR 185	1
RRR 60	2	RRR 123	2	RRR 186	1
RRR 61	2	RRR 124	0	RRR 187	0
RRR 62	1	RRR 125	1	RRR 188	1
RRR 63	2	RRR 126	1	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 %.

While in secondary screening, all the *Bacillus spp.* i.e.which found highly antagonistic during the primary screening, were selected and screened again with *Sclerotium rolfsii* by dual culture method. Out of these twenty *Bacillus spp.*, only *Bacillus spp.* RRR15, found highly effective in controlling the phytopathogen, *Sclerotium rolfsii in vitro*, in dual culture method (Photo Plate 3.0) These *Bacillus spp.*RRR15effectively killing the growth of phytopathogen, *Sclerotium rolfsii* whose percent inhibition was 87.5as shown in Table 3.1

Table 3.1: *In Vitro* Secondary Screening for efficient Microbiological control Agent, *Bacillus spp.***RRR15 selected during primary screening against** *Sclerotium rolfsii* Sacc

Isolates of *Bacillus spp.*	**Radial growth of the pathogen in the opposite direction of the antagonist (a control value) R1 (mm)**	**R2 is radial growth of the pathogen in the direction towards the antagonist (an inhibition value) R2(mm)**	**Percent Inhibition (%) of *Sclerotium rolfsii***
RRR 15	45	05	87.5

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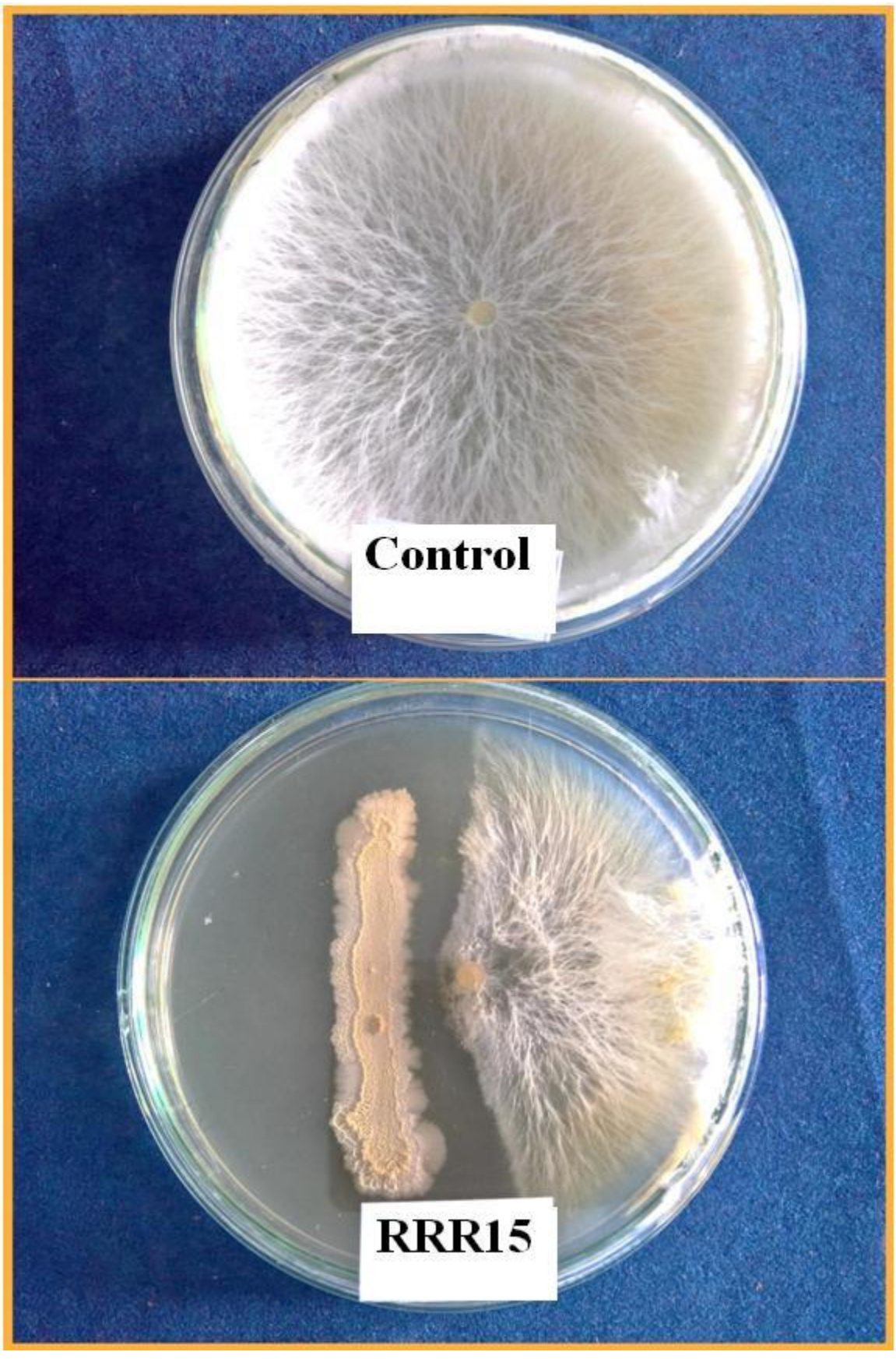


Photo Plate 3.0: - *In Vitro* Secondary Screening of Efficient*Bacillus spp.* RRR15 against*Sclerotium rolfsii*in Dual Culture Method

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. rolfsiiin vitro* in dual culture assay. Among these seven 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 tested isolate against*S. rolfsii*.

When all these results were compared with our results, our findings showed that *Bacillus spp.*RRR15 significantly preventing mycelial growth of *Sclerotium rolfsii* in *dual culture technique* with inhibition percentage of 87.5. Our findings were far better than these previously recorded results [Keyser and Ferreira, (1988), Gomashe *et al.,* (2014),Shifa*et al.,* (2015)Rajkumar *et al.,* (2018)].

3.3 Identification of *Bacillus spp*. RRR15:

16S rRNA sequencing and Phylogenetic analysis identified RRR15 as *Bacillus amyloliquefaciens* RRR15(Fig. 3.0). The 16S rRNA sequence has been deposited in Genbank of National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine8600 Rockville Pike, Bethesda MD, 20894 USA with accession no. MN744706.

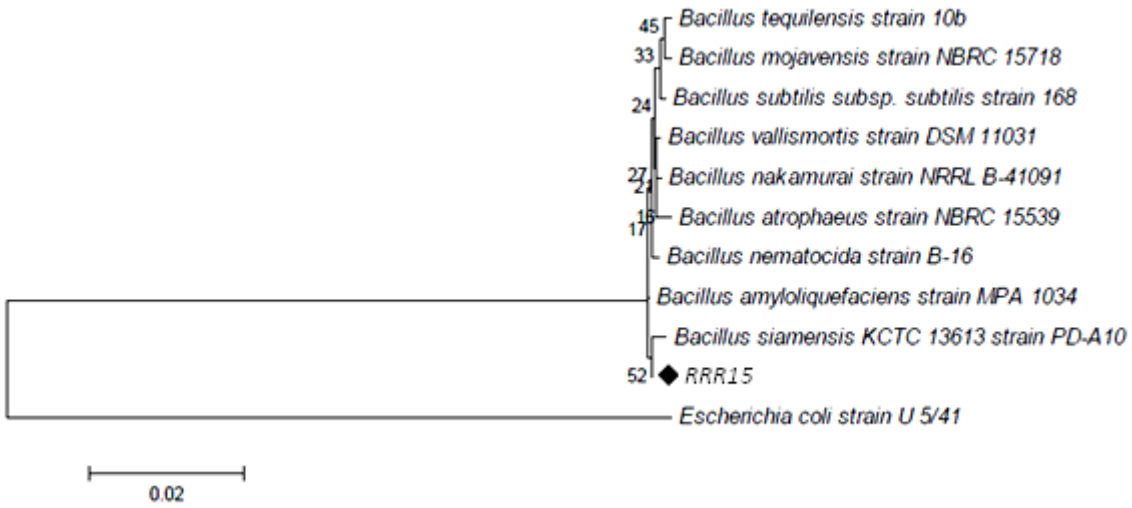


Figure 3.0:Phylogenetic tree of *B.amyloliquefaciens strain* RRR15 **with closely related species after BLAST search of 16S rRNAsequences.The bootstrap values are indicated at the branch points.**

4.0 Acknowledgment:

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.) Dist. Parbhani.

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