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Efficacy of different bioagents against stem rot of groundnut incited by *Sclerotium rolfsii* under *in vitro* conditions

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Abstract
Stem rot caused by *Sclerotium rolfsii* considered as one of the most devastating disease of groundnut. The main aim of this study was to find out the potentiality of various fungal and bacterial bioagents *viz*., *Trichoderm*a *harzianum* (BKN), *T. viride* (BKN), *T. harzianum* (JPR), *T. viride* (Tv-1), *T. atroviride* (Ta-7), *T. atroviride* (Ta-15), *T. longibrachiatum, P. fluorescens* (Pf-BKN), *P. fluorescens* (Pf-1), *P. fluorescens* (Pf-2), *B. subtilis* (Bs-BKN), *B. subtilis* (Bs-1) and *B. subtilis* (Bs-2) against the *S. rolfsii*. Among the fungal bioagents, maximum growth inhibition of *S. rolfsii* was recorded with *T. harzianum* (Th-BKN) (88.66%) followed by *T. viride* (Tv-BKN) (85.27%) and *T. harzianum* (Th-JPR) (84.93%). Similarly, *B. subtilis* (Bs-BKN) (70.54%) and *P. fluorescens* (Pf-BKN) (63.79%) also gave maximum mycelial growth inhibition of *S. rolfsii* under *in vitro* conditions. Culture filtrate of different bioagents at the concentration of 1, 2.5 and 5% were tested and the maximum growth inhibition of *S. rolfsii* was recorded with *T. harzianum* (Th-BKN) 88.64 per cent at 5 per cent concentration followed by *T. viride* (Tv-BKN) (84.28%). The results indicated that *T. harzianum* (BKN), *T. viride* (BKN), *T. harzianum* (JPR), *P. fluorescens* (Pf-BKN), and *B. subtilis* (Bs-BKN), potentially control the growth of *S. rolfsii* and could be used for further exploitation under field conditions.

Keywords: *Sclerotium rolfsii*, fungal bioagents, bacterial bioagents and management

Introduction
Groundnut (*Arachis hypogea* L.) is commonly called peanut, goober pea, jack nut, manila nut, pignut and monkey nut (Rathnakumar, *et al*., 2013) [31]. It is known as 'king of oil seeds (Aycock, 1966) [5]. It belongs to the family of leguminaceae and originated from South America. It was cultivated as early as 1000 B.C. (Wiess, 2000) [37]. Groundnut is grown on 23.25 million hectares worldwide with a total production of 40.08 million metric tonne and productivity of 1676 kg ha^{-1} (Anonymous, 2015a) [1]. The country major groundnut producing state has brought India's estimated *kharif* groundnut output down 24 per cent (Anonymous, 2015b) [2]. In Rajasthan, it is an important oilseed crop of the semi-arid tropics, cultivated in about 5.16 lakh hectares with an annual production of 10.48 lakh tones and productivity of 2029 kg ha^{-1} (Anonymous, 2015c)[3]. On groundnut, more than 70 diseases have been reported (McDonald *et al*., 1985; Lukose *et al*., 2008) [23, 25]. Stem rot (*Sclerotium rolfsii* Sacc.), Collar rot (*Aspergillus niger* Van Teighem), early leaf spot (*Cercospora arachidicola* Hori), late leaf spot (*Cercospora personatum* Berk and Curt), root rot (*Rhizoctonia solani* Kuhn), and rust *(Puccinia arachidis* Speg.) are the major diseases of groundnut prevalent in India (Faujdar and Oswalt, 1992) [13]. Among the diseases the stem rot disease is a notorious phytopathogen causing considerable yield losses under congenial weather conditions (Desai and Bagwan, 2005) [12]. *Sclerotium rolfsii* is seed and soil borne in nature and have a wide host range more than 500 species in 100 families including groundnut, green bean, lima bean, onion, garden bean, pepper, potato, sweet potato, tomato and water melon (Aycock, 1966) [5]. Stem rot is more extensive in the *kharif* than the *rabi* and summer seasons and causes more damage in sandy loam and medium black soil. Stem rot incidence occurs in more than 5 lakh/ha of groundnut area and causes severe damage during any stage of crop growth and yield losses reach over 80% in heavily infected fields (Mayee and Datar 1988; Mehan and McDonald 1990) [24, 26].

Management of this pathogen with synthetic fungicides is not very effective as it provide protection for limited period and lead to environmental pollution. The continuous use of traditional fungicides may cause bioaccumulation of the toxic residues and development of

fungicide resistant in the pathogen. Increased public concern about pesticide utilization and the health hazards necessitates the exploitation of alternative methods for disease control that is eco-friendly and effective is only biological control for fungal plant pathogens. Now a day's research on disease management all over the world is mostly towards biological control. In the last three decades, a lot of researches have been carried out on the antagonistic nature of several fungal and bacterial biocontrol agents (Kishore *et al*., 2005; Couillerot *et al*., 2008) [8, 9]. Blakeman and Fokkema (1982) [20] reported that *Trichoderma* species are the well-known antagonists, particularly in the soil and that they are involved in competition, antibiosis and hyperparasitic interactions, which makes them the most effective biocontrol agents even on foliar surfaces. Management of *Sclerotium rolfsii* under *in vitro* conditions through fungal (*Trichoderma* species) and bacterial (*P. fluorescens* and *B. subtilis*) antagonistic microorganisms has been reported (Biswas *et al*., 2000; Girija and Umamaheswaran, 2003; Arunasri *et al.*, 2003; Srinivasulu *et al*., 2005; Joshi *et al*., 2010; and Prasad *et al.*, 2017) [4, 7, 14, 17, 29, 33]. These bioagents are less detrimental, eco-friendly and safer than synthetic pesticides (Hashim and Devi, 2003) [15]. The fungal and bacterial bioagents are the potent biocontrol agents for the management of soil borne diseases. These bioagents may help in reducing the excess use of hazardous chemical pesticides and can improve economic conditions of farmers. Keeping above the facts in mind the main objectives of the present study was to investigate the potentiality of different bioagents under *in vitro* conditions and could be used for further exploitation under field condition for management of *S. rolfsii*.

Materials and Methods

Groundnut stem portion affected by stem rot was collected from Research Farm, College of Agriculture, SKARU, Bikaner. The pathogen was isolated from diseased stems on Potato Dextrose Agar (PDA) and purified by using tip of single hypha. The hyphal suspension of the respective isolate was prepared in sterilized distilled water, so as to obtain 5-6 hypha per microscopic field. The hyphal suspension was spread over the surface of sterilized 2 per cent plain agar medium in Petri dishes and incubated at 28±2 ^{0}C for 24 hours. The single hyphal piece was observed under low power objective and cut through dummy objective. Such pieces were transferred separately on Potato Dextrose Agar (PDA) slants with the help of an inoculating needle and incubated at 28±2 ^{0}C for 24 hours. Pure culture was maintained and stored in refrigerator at 5 ºC for further studies. Similarly, seven fungal and five bacterial bio-agents were isolated from groundnut rhizosphere *viz*., *Trichoderma harzianum* (BKN), *T. viride* (BKN), *T. harzianum* (JPR), *T. viride* (Tv-1), *T. atroviride* (Ta-7), *T. atroviride* (Ta-15), *T. longibrachiatum*, *P. fluorescens* (Pf-BKN), *P. fluorescens* (Pf-1), *P. fluorescens* (Pf-2), *B. subtilis* (Bs-BKN), *B. subtilis* (Bs-1) and *B. subtilis* (Bs-2) by using serial dilution method (Koch, 1883)[21]. Pure culture of all bioagents were maintained and stored in refrigerator at 5 ºC for further investigation.

a. Evaluation of fungal antagonists against *S. rolfsii* under *in vitro* conditions: Dual culture method (Morton and Strouble 1955) [28] was followed in order to ascertained the antagonistic capacity of *Trichoderma* species. One mycelial disc (5 mm diameter) of the pathogen and each antagonist were kept on the surface of potato dextrose agar medium in Petri dishes at 5 cm distance. The inoculated Petri dishes were incubated at 28±2 ^{0}C for 24 hours. Three replications were kept for each fungal antagonist. In case of control, the Petri dishes were inoculated with mycelial discs of the test pathogen only. The mycelial growth of test pathogen was measured after seven days of inoculation. The inhibition of mycelial growth of the pathogen was calculated by using the formula suggested by (Dennis and Webster, 1971) [11]; [PGI= C – T / C] × 100, Where, PGI = Per cent growth inhibition; C = Mycelial growth of *S. rolfsii* in control (mm); T = Mycelial growth of *S. rolfsii* in presence of antagonist (mm).

b. Evaluation of bacterial antagonists against *S. rolfsii* under *in vitro* conditions: Paper disc method (Loo *et al*., 1945) [22] was followed to ascertain the antagonistic potentiality of bacterial bioagents. Circular discs (5 mm dia.) of Whatman filter (No. 42) were cut and dipping in suspension of bacterial antagonists then placed 1 cm inward from the periphery of Petri dishes at four equidistance places, having in the centre the inoculum of pathogen (*S. rolfsii*). The inoculated dishes were incubated at 28±2 ^{0}C for 24 hours and observations were recorded. Three replications were kept for each bacterial antagonist.

c. Effect of different culture filtrates of bioagents on mycelial growth of *S. rolfsii* under *in vitro* conditions: This experiment was conducted to test the effect of culture filtrates of the four bioagents as described by Upadhyay and Rai (1987) [35]. The antagonists were grown in liquid media *i.e.* Potato Dextrose Broth for *T. harzianum* (Th-BKN), *T. viride* (Tv-BKN) and *T. atroviride* (Ta-7), King's B broth and Nutrient Broth for *P. fluorescens* (Pf-BKN) and *B. subtilis* (Bs-BKN), respectively. The antagonists were incubated at desired temperature in BOD incubator. In case of *Trichoderma* spp., the seven days old cultures were first filtered through double layered cheese cloth followed by filtering through Whatman (No. 1) filter paper. The culture filtrate obtained was centrifuged at 10000 rpm at 4 ^{0}C for 15 minutes. The supernatant was then passed through bacterial proof filter and stored in refrigerator. The two bacterial antagonists *i.e. P. fluorescens* (Pf-BKN) and *B. subtilis* (Bs-BKN) were raised in Kings' B broth (King *et al*., 1954) [18] and Nutrient Broth media, respectively, for 72 hours at desired temperature in BOD incubator. The broth media containing the bacterial growth were centrifuged at 10000 rpm for 15 minutes in 4 ^{0}C and the supernatant was passed through bacteria proof filter and stored in refrigerator for further studies. In order to study the inhibition potentiality, the respective supernatants were added to Potato Dextrose Agar medium at 1, 2.5 and 5 per cent concentrations at the time of pouring of the media in Petri dishes. Mycelial discs (5 mm diameter) taken from periphery of actively growing culture of *S. rolfsii* was placed at the centre of Petri dishes containing PDA medium previously amended with the respective supernatants. In case of control no supernatant was added to PDA. Three replications were kept for each type of culture filtrate. The inoculated Petri dishes were incubated at 28±2 ^{0}C. Mycelial growth of *S. rolfsii* was recorded after 7 days of incubation. The inhibition of mycelial growth by the respective fungal and bacterial antagonists was calculated by using the formula suggested by (Dennis and Webster, 1971) [11].

Results and Discussion

a). Evaluation of fungal antagonists against *S. rolfsii* under *in vitro* conditions: Efficacy of seven fungal bio-agents *viz.*, *T. harzianum* (Th-BKN), *T. harzianum* (Th-JPR), *T. viride* (Tv-

BKN), *T. viride* (Tv-1), *T. atroviride* (Ta-7), *T. atroviride* (Ta-15) and *T. longibrachiatum* (Tl-2) were tested against *S. rolfsii*. All the tested fungal bio-agents significantly inhibited the mycelial growth of *S. rolfsii.* The results given in Table 1 revealed that *T. harzianum* (Th-BKN) (88.66%) found superior over all the bioagents. *T. viride* (Tv-BKN) (85.27%) and *T. harzianum* (Th-JPR) (84.93%) were also effectively suppressed the mycelial growth of *Sclerotium rolfsii* followed by *T. viride* (T.v.-1) (84.33%) and *T. atroviride* (Ta-7) (81.00%). *T. harzianum* (Th-JPR) and *T. viride* (Tv–1) significantly suppressed the growth of the test pathogen and found statistically at par to each other. Two antagonists *T. atroviride* (Ta-15) (70.48%) and *T. longibrachiatum* (Tl-2) (57.67%) were observed less inhibitory. Biswas *et al*. (2000) [7] tested the efficacy of 11 isolates of *Trichoderma harzianum* under *in vitro* conditions and reported that the three isolates, viz.., T8, T10 and T2 were found effective and overgrew the *Sclerotium rolfsii* of groundnut up to 92, 85 and 79 per cent respectively that are supported to our investigation. Similarly, Bagwan (2011) [6] screened forty six isolates of *Trichoderma spp.* belonging to viz. *viride, hamatum*, *ressei* and *koningi species* groups against *Sclerotium rolfsii Sacc, Aspergillus niger van Teighem and A.flavus*, the causal agents of stem rot, collar rot and alfaroot of groundnut respectively. The maximum mycelial growth inhibition of *Sclerotium rolfsi* was recorded with the isolates of T005, T043, T095, T49, T126, T144, T166, T191, 250, 390 and T425 in dual culture techniques. Prashant Kumar *et al*. (2011) [30] evaluated four bioagents against *S. rolfsii* causing root rot of chilli (*Capsicum annum* L.) under *in vitro.* Among the four bioagents tested *Trichoderma harzianum* recorded maximum growth inhibition (77.82%) of *S. rolfsii,* followed by *T. viride* (66.06%) that are also in agreement with our study. Ritesh Kumar *et al*., in 2012 [32] studied the antagonistic potential of *Trichoderma* species isolated from two different soils i.e alfisols and inceptisols and they found that the alfisol isolates showed higher potential antagonism against *S. rolfsii* with per cent inhibition of 44.67 and 47.88 as compared to inceptisol isolates with inhibition percentage of 3.97, 7.97 and 28.72 respectively. Similarly, Jabbar *et al*. (2014) [16] reported that *T. harzianum*-55 IIHR showed highest inhibition (70%) against *S. rolfsii* followed by *T. harzianum* NBAII (63%), *T. viride* IIHR (59%) whereas *T. harzianum* IIHR (37%), *T. viride* GKVK (36%) and *T. harzianum* GKVK (32%) did not show much variation in the inhibition percentage that also corroborated to our study.

b). Evaluation of bacterial antagonists against *S. rolfsii* under *in vitro* conditions: The Efficacy of six bacterial bio-agents *viz., Pseudomonas fluorescens* (Pf-BKN), *P. fluorescens* (Pf-1), *P. fluorescens* (Pf- 2), *B. subtilis* (Bs-BKN), *B. subtilis* (Bs-1) and *B. subtilis* (Bs-2) were evaluated against *S. rolfsii*. The mycelial growth of *S. rolfsii* was suppressed by all the tested bacterial bio-agents. The results revealed that the *B. subtilis* (Bs-BKN) (70.54%) were relatively more inhibitory to *S. rolfsii* followed by *P. fluorescens* (Pf-BKN) (63.79%). The strains *B. subtilis* (Bs-1) (60.43%) and *P. fluorescens* (Pf-1) were relatively less effective. Among the tested strains *B. subtilis* (Bs-2) (45.52%) and *P. fluorescens* (Pf-2) (42.50%) were least inhibitory to test pathogen (Table 2). Arunasri *et al.* (2003) [4] reported that *in vitro* efficacy of antagonistic mycoflora and a bacterium against *S. rolfsii* was evaluated by using dual culture technique. *Trichoderma* isolate-1 (T1) and *Pseudomonas* sp. (B1) were found to be potential native antagonists that inhibited growth and sclerotial population of *S. rolfsii* to a maximum of 69.76%, 90.86% and 43.10%, 71.00% respectively that is corroborated to our study. Similarly, Girija and Umamaheswaran (2003) [14] observed the effect of the bioagents against basal rot pathogen of balsam (*S. rolfsii*) under *in vitro* conditions and showed that *Trichoderma virens* gave maximum inhibition (65.8%) followed by 45.8% inhibition by B1 isolate of *Bacillus* sp. Vishwanath *et al*. (2012) [36] were tested *Pseudomonas fluorescens, Bacillus subtillis*, *Pseudomonas* sp.-I, *Bacillus* sp.-I and *Bacillus* sp.-II biocontrol agents against *Sclerotium rolfsii* in dual culture technique and they found that all the bioagents were overgrew the pathogen up to 76.2, 88.8, 80.01, 35.9 and 77.8 per cent respectively, that are also in agreement with our investigation. Suneeta *et al.* (2016) [34] observed that, *in vitro* screening of 26 isolates of *Bacillus* spp. against collar rot pathogen. Among them, five strains of *Bacillus* spp. showed highest antagonistic activity against *S. rolfsii* that also supported to our findings. Similarly, Prasad *et al*. (2017) [29] evaluated bio-agents potentiality of twenty-four fungal and twelve bacterial bio-agents against *Sclerotium rolfsii*. They found that fungal antagonist *T. harzianum-1* and bacterial bio-agents *P. fluorescens* (Pf-3) significantly inhibited the mycelial growth of *Sclerotium rolfsii* under *in vitro* conditions.

c). Effect of different culture filtrates of bioagents on mycelial growth of *S. rolfsii* under *in vitro* conditions: Effect of five bio-agents culture filtrate *viz., T. harzianum* (Th-BKN), *T. viride* (Tv-BKN), *T. atroviride* (Ta-7), *P. fluorescens* (Pf-BKN), and *B. subtilis* (Bs-BKN) were studied against mycelial growth of *S. rolfsii* at three different concentrations i.e. 1, 2.5 and 5 per cent respectively. The results given in Table 3 revealed that culture filtrate of the respective bio-agents inhibited the mycelial growth of *S. rolfsii* to various extents. The maximum inhibition of the pathogen was recorded with culture filtrate of *T. harzianum* (Th-BKN). The growth inhibition by *T. harzianum* (Th-BKN) was 88.64 per cent followed by *T. viride* (Tv-BKN) (84.28%) at 5 per cent concentration. The culture filtrate of *P. fluorescens* (Pf-BKN) was also checked the growth of pathogen. The culture filtrates of *T. atroviride* (Ta-7) and *B. subtilis* (Bs-BKN) were less inhibitory as compared to rest of the three antagonists. It was also recorded that the growth of the pathogen decreased with the increase in concentration of culture filtrate of all the five bio-agents tested. However, decreased concentrations were less inhibitory to the growth of *S. rolfsii.* Kapil and Kapoor (2005) [18] tested the volatile and non-volatile metabolites of bioagents *viz. T. harzianum, T. viride and T. atroviride* which significantly reduced the mycelial growth and germination of *Sclerotinia sclerotiorum* that is supported to our findings. Mishra *et al.* (2011) [27] reported the efficacy of culture filtrate of *T. viride* Tr 8 against *M. phaseolina* and other soil borne pathogens under *in vitro* conditions that also suppressed the growth of test pathogen. Similarly, Darvin *et al.* (2013) [10] observed the suppressive effect of culture filtrate metabolites of three fungal bio-agents against mycelial growth of *S.rolfsii* causing stem rot of groundnut. The results from this investigation revealed that *T. viride* (TvL), *T. harzianum* 4 (Th 4) and *T. harzianum* 14 (Th14) isolates were found more effective and showed lowest radial growth of 3.50 cm and highest per cent inhibition (56.25%) of *S. rolfsii*. The highest radial growth (4.23 cm) and lowest per cent inhibition (47.1%) were recorded with *T. longibrachiatum* (Tl2). Results from non-volatile assay indicated that irrespective of concentration, culture filtrate of *T. viride* (TvL) was found to be most effective, recorded lowest radial growth and highest per cent inhibition followed by *T. harzianum* (Th14) and *T. harzianum*

(Th4) which also corroborated to our study. Thus, in our study we have found that bioagents are eco-friendly, cost effective free from environmental pollution and very effective in controlling of stem rot disease as compared to other methods. Even theses bioagents are good alternative for the farmers who totally rely on chemicals. They work as a plant growth promoting rhizobacteria (PGPR) that indirectly increase the growth and yield of crops. These bioagents can be used as a component of integrated disease management (IDM) for management of *S. rolfsii* and against other deadly soil borne pathogens.

Based on present investigation results it can be conclude that fungal bioagents, *viz*., *T. harzianum* (Th-BKN) (88.66%) *T. viride* (Tv-BKN) (85.27%) and *T. harzianum* (Th-JPR) (84.93%) followed by *B. subtilis* (Bs-BKN) (70.54%) and *P. fluorescens* (Pf-BKN) (63.79%) bacterial bioagents were found very effective and gave maximum mycelial growth inhibition of *Sclerotium rolfsii* under *in vitro* conditions. Similarly, in cultural filtrate, the maximum growth inhibition by *T. harzianum* (Th-BKN) was 88.64% at 5 per cent concentration followed by *T. viride* (Tv-BKN) (84.28%) against *S. rolfsii*.

Table 1: Efficacy of fungal bio-agents on mycelial growth of *S. rolfsii*

Bio-agents	Mycelial growth (mm)	Inhibition (%)
Trichoderma harzianum (BKN)	10.21 (18.63)*	88.66
T. viride (BKN)	13.26 (21.35)	85.27
T. harzianum (JPR)	13.56 (21.61)	84.93
T. viride (Tv-1)	14.10 (22.06)	84.33
T. atroviride (Ta-7)	17.10 (24.43)	81.00
T. atroviride (Ta-15)	26.30 (30.85)	70.78
T. longibrachiatum	38.10 (38.12)	57.67
Control	90.00 (71.57)	-
S.Em ± CD (P=0.05) CV (%)	1.34 4.12 7.21	

*Figures in parentheses are angular transformed values

Table 2: Efficacy of bacterial bio-agents on mycelial growth of *S. rolfsii*

Bio-agents	Mycelial growth (mm)	Inhibition (%)
P. fluorescens (Pf-BKN)	32.59 (34.81)*	63.79
P. fluorescens (Pf-1)	44.30 (41.73)	50.78
P. fluorescens (Pf-2)	51.75 (46.00)	42.50
B. subtilis (Bs-BKN)	26.51 (30.99)	70.54
B. subtilis (Bs-1)	35.61 (36.64)	60.43
B. subtilis (Bs-2)	49.30 (44.60)	45.52
Control	90.00 (62.03)	-
SEm ± CD (P=0.05) Cv(%)	1.65 5.09 5.83	

*Figures in parentheses are angular transformed values

Table 3: Efficacy of culture filtrate of bio-agents on mycelial growth and per cent inhibition of *S. rolfsii*

Bio-agents	Mycelial growth and per cent Inhibition					
	1 per cent		2.5 per cent		5 per cent	
Trichoderma harzianum (Th. BKN)	14.27 (22.17)*	84.14	12.29 (20.50)	86.34	10.22 (18.62)	88.64
T. viride (Tv-BKN)	18.52 (25.47)	79.42	16.65 (24.06)	81.5	14.14 (22.07)	84.28
T. atroviride (Ta-7)	19.87 (26.45)	77.92	17.82 (24.95)	80.2	15.78 (23.39)	82.46
P. fluorescens (Pf-BKN)	28.81 (32.44)	67.98	27.56 (31.65)	69.37	24.49 (29.64)	72.78
B. subtilis (Bs-BKN)	24.90 (29.91)	72.33	22.12 (28.01)	75.42	19.94 (26.19)	77.84
Control	90 (71.59)	-	90 (71.59)	-	90 (71.59)	-
S.Em ±	0.46		0.43		0.41	
CD (P=0.05)	1.47		1.37		1.32	
CV (%)	3.32		2.89		2.62	

*Figures in parentheses are angular transformed values

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