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Studies on the biological methods for the management of Coffee (*Coffea arabica L.*) collar rot (*Rhiozoctonia solani* khun) by Coffee bio-capsules

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ABSTRACT

Coffee is an important plantation crop in India. *Arabica* and *Robusta* coffee are susceptible to fungal disease. Coffee plants propagated through seeds. *Rhiozoctonia solani* khun is known to cause collar rot/damping off of coffee seedlings in the nursery beds and lead to 10-25 % mortality of seedlings under favourable conditions. For effective management of this disease by bio capsule methods the present study was carried out, ten *Rhiozoctonia solani* khun isolates were collected from different regions. The pathogenicity effect to produce collar rot symptoms showed that R.s tn 4 is high virulent compared to other isolates during the period of April- June were noticed. In case of bio control agents such as *Bacillus subtilis* (58.65%) inhibition found to be maximum mean per cent inhibition compared to untreated control (70.50%)

Key words*: Rhizoctonia solani khun, Bacillus subtlis, Collar rot, Pathogenicity test and coffee biocapsules.*

Introduction

The area of Coffee cultivation in Tamil Nadu is around 33,904 ha with an average production of about 18,289 metric tonnes and productivity of 539 kilos per ha. However, pests and diseases are the major hurdles which affect the production and productivity. Among them *Rhizoctonia solani* khun is the major constraint in coffee production which causes collar rot and it is also widespread in traditional coffee growing areas and the disease incidence is as high as 25-30 per cent for month of April- June (Sudha *et al.,* 2020). While coffee seed require high moisture and temperature for germination, this condition is also ideal for growth of *Rhizoctonia solani khun* in the seedbeds. Valdez and Acedo (1963) reported that moisture level in coffee nursery beds has direct influence on collar rot incidence. The common practice of watering the seedbeds twice daily for 10 days and once a day thereafter predisposes the seedlings to *Rhizoctonia solani khun* infection collar rot or damping off disease occurs on 40 to 50 days old tender coffee seedlings in the coffee nursery. Sometimes, the collar rot pathogen attacks coffee seeds sown on the seed beds and prevent its germination. However, the pathogen fails to establish on coffee seedlings when the stem at the collar region turn brown and become harder. It is a soil inhabiting fungus with a wide host range including coffee. The pathogen is capable of surviving in the soil for many

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months in the form of sclerotia under favourable condition it's germinate into mycelia and infect coffee seedlings. Two phases of this disease are described as under: 1) Pre emergence stage: The pathogen invades embryo and endosperm of the nursery seed beds; consequently, seeds start rotting, disintegrate and fail to germinate. 2) Post emergence stage: Seedlings show brown to black discoloration at the collar region of the stem leading to rotting of the tissues. Growing tip of the seedlings wilts, collapses and dies (Sudhakar and Bhat, 2016). The application of fungicides is not economical in the long term because they pollute the environment leave harmful residues and lead to development of resistant strains of the pathogen and suppress the beneficial microorganisms in the soil (Vinale *et al*., 2008). However, use of biological control is safe non-hazardous for human, farm animals and avoids environmental pollution. The Application of biological controls using antagonistic microorganisms has proved to be successful for controlling various plant diseases in many countries (Janisiewicz 2000).

Materials and Methods

A *Coffea arabica L* variety namely Chandragiri is widely grown cultivar in India used in the present study. The seeds were superficially disinfected with 5% sodium hypochlorite for 5 min, rinsed thrice with sterile distilled water (SDW) and allowed to dry at room temperature. Seeds were then sown in plants containing pot mixture (6:2:1) previously sterilized at 110°C for one hour and kept under greenhouse conditions for 45 days.

Pathogen culture and inoculum preparation

Ten isolates of *Rhizoctonia solani khun.* recovered from diseased coffee plants showing collar rot symptoms and collected from different sites (Tamil Nadu, Karnataka and Kerala) were used in the present study (Table 1). Coffee isolates were characterized by morphological study and colony characterizes such as *sclerotial* formation. Isolates were grown on Potato Dextrose Agar (PDA) medium amended with streptomycin sulfate (300 mg/l) and maintained in the dark for 7 days at 25 °C. To prepare pathogen inoculum, *Rhizoctonia solani* khun. mycelia were collected from 5- 7 day-old cultures grown on PDA medium and homogenized in 0.5 L of SDW with an electric mixer for 5 min. The resulting mycelial fragments served for substrate inoculation. Pathogen inoculum were added and mixed thoroughly with the culture substrate before planting. 5% inoculum was used in this study.

Plate 1. Mother culture of *R. solani* Kuhn (Rs.Tn.4)

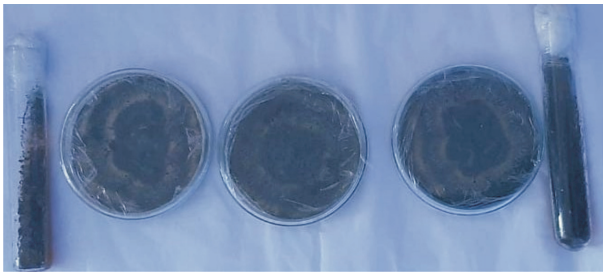

Plate 2. Mother culture of *Trichoderma harzianum (Native)*

Table 1. *Rhizoctonia solani* Kuhn isolates, cultivar and places used in the studies

Sl. No.	Isolates	Original host	Plant cultivar	Original site	Longitude °C	Latitude °C
1	$R._S - tn_1$	*Coffee arabica*	Chandragiri (Sln. 13)	RCRS, Thandigudi, Tamil Nadu	77.64 °C	10.30 °C
2	$R._S - tn_2$	*Coffee arabica*	Chandragiri (Sln. 13)	RCRS, Thandigudi, Tamil Nadu	77.64 °C	10.30 °C
3	$R._S - tn_3$	*Coffee arabica*	Chandragiri (Sln. 13)	RCRS, Thandigudi, Tamil Nadu	77.64 °C	10.30 °C
4	$R._S - tn_4$	*Coffee arabica*	Chandragiri (Sln. 13)	RCRS, Thandigudi, Tamil Nadu	77.64 °C	10.30 °C
5	$R._S - ka_1$	*Coffee arabica*	Selection. 9	Chikmagaluru, Karnataka	75.77 °C	13.32 °C
6	$R.S - ka_2$	*Coffee arabica*	Selection. 9	Chikmagaluru, Karnataka	75.77 °C	13.32 °C
7	$R.S - ka_3$	*Coffee arabica*	Selection. 9	Chikmagaluru, Karnataka	75.77 °C	13.32 °C
8	$R.S - ka_4$	*Coffee arabica*	Selection. 9	Chikmagaluru, Karnataka	75.77 °C	13.32 °C
9	$R.S - kl_1$	*Coffee arabica*	Robusta coffee	Wyanad, Kerala	75.86 °C	11.84 °C
10	$R.S - kl_2$	*Coffee arabica*	Robusta coffee	Wyanad, Kerala	75.86 °C	11.84 °C

Fungal and bacterial bio control agents

One fungal antagonist, namely *Trichoderma harzianum* and two antagonists namely *Pseudomonas fluorescens* and *Bacillus subtilis* were selected from coffee rhizosphere soils were collected. These bio-agents, originally recovered from Coffee rhizosphere soils were previously shown effective against several soil borne plant pathogens. Fungal suspensions were prepared by scraping off mycelium from 7-day-old cultures grown on PDA medium, homogenized with SDW, and then filtered through two-layers of muslin. The resulting conidial suspension was adjusted to 10^7 CFU/ml using a colony counter Malassez hemocytometer. Two bacterial isolates belonging to *Pseudomonas florescence, Bacillus subtilis* one fungal isolates belonging to *Trichoderma harzianum* were used in this study (Table 2).

Rhizobacterial stock cultures were maintained on

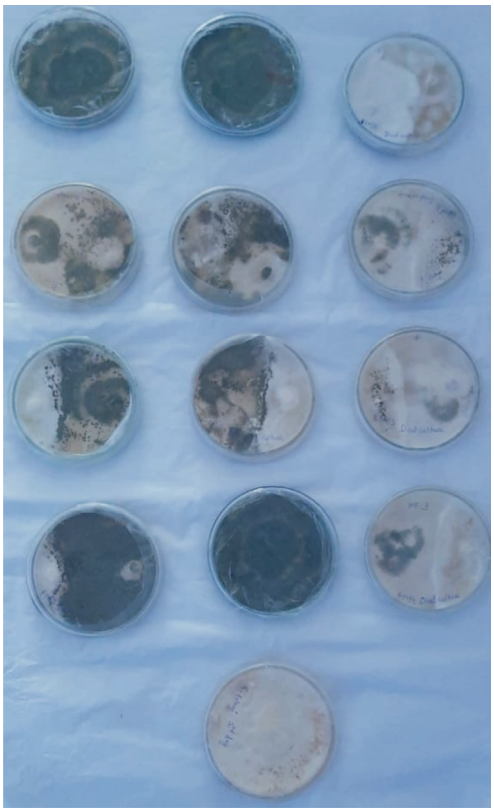

Plate 3. Dual culture technique against *R. solani Kuhn*

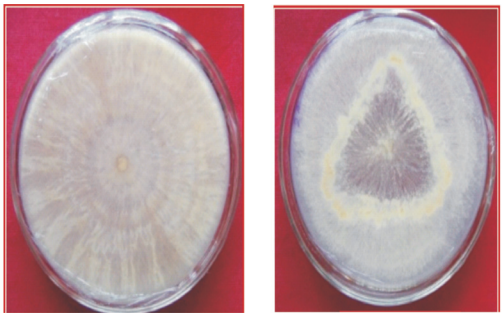

Plate 4. Dual culture technique against *Bacillus subtilis* (Native)

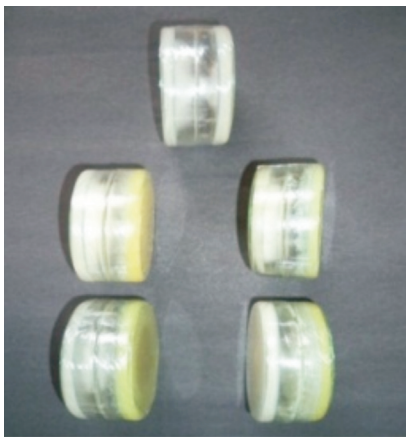

Plate 5. Determination of *Trichoderma Harzianu* virulence

Plate 6. *Trichoderma* Solid formulation fluorescens

Plate 7. Determination of *Pseudomona*

Kings B and Nutrient Agar (NA) medium supplemented with 40% glycerol and stored at 4 °C. Before use, bacterial isolates were grown on NA and Kings B incubated at 25 °C for 48 h. Fungal antagonist culture were maintained on PDA medium and stored at 25 °C for 7 days. Bacterial and fungal cell suspensions of *Trichoderma harzianum, Pseudomonas fluorescens and Bacillus subtilis* used for *in vitro* and *in vivo* bioassays were prepared by scraping bacterial colonies and spore mate previously grown in NA/PDA for 48 hours to 7 days in Sterile Distilled Water and adjusted to 10^6 cells/ml.

To test the ability of ten *Rhizoctonia solani* khun. isolates to cause pre- and post-emergence damping-off disease, disinfected coffee seeds were sown in polybag (5/8 inches) filled with sterilized pot mixture mixed with *Rhizoctonia solani* infected substrate at the rate of 1:3 (v/v). Seeds sown in non-infected pot mixture were used as uninoculated control. One seed was used for each individual treatment. The percentage of seed germination and seedling emergence were determined after 45 days of incubation under net house conditions. The ten *Rhizoctonia solani* khun. Isolates were also tested on coffee seedlings for their ability to cause collar rot disease. Forty days old coffee topee stage seedlings were inoculated by root dipping for 30 min in the fungal suspensions of each *Rhizoctonia solani khun.* Isolate (mycelial fragments) prepared as previously described. Seedlings which roots were dipped in SDW only served as uninoculated control. All seedlings were then transplanted into pot mixture (6:2:1) previously were grown under net house conditions for 90 days. At the end of the experiment, coffee plants were uprooted and washed to eliminate the adhering soil partials. Plant height and aerial parts and roots fresh weights were recorded. Disease severity was estimated based on the density of *Rhizoctonia solani khun.* Lesions formed on collar rot according to a 0-5 scale, where 0=absence of visible lesions in the collar; 1=1 to 25% of the collar covered with lesions; 2=26 to 50% of the collar covered with lesions; 3=50 to 75% of the collar covered with lesions; 4=large lesions (> 75%) and 5=dead plant. Pathogen re-isolations were performed from roots and collar rot of inoculated plants to confirm Koch postulate.

In vitro antagonism assay

Dual culture plate assays were performed in 90-mm Petri plates containing PDA to test the ability of fungal and bacterial agents to inhibit *Rhizoctonia solani*

Table 2. Pathogenicity test – Biometric observation and collar rot severity of coffee seedlings

Treatment	Plant height (cm)			Shoot length (cm)			Root length (cm)			Disease Incidence (%)		
	Topee	BF	3m	Topee	BF	3m	Topee	BF	3m	Topee	BF	3m
R. S -tn 1	11.25^{bcd}±0.17	16.00^{a}±0.24	21.50^{a}±0.33	4.75^{b}±0.07	5.50^{bc}±0.09	6.50^{b}±0.10	6.50^{b}±0.10	10.50^{b}±0.16	15^{b}±0.23	6.50^{b}±0.10	10.50^{b}±0.16	15^{b}±0.23
R.S - tn 2	11.60^{bc}±0.18	15.25^{b}±0.23	20.50^{b}±0.31	4.50^{c}±0.07	5.75^{b}±0.09	6.50^{b}±0.10	7.10^{cd}±0.11	9.50^{d}±0.15	14^{e}±0.21	7.10^{cd}±0.11	9.50^{d}±0.15	14^{c}±0.21
R.S - tn 3	10.55^{ef}±0.16	15.00^{b}±0.23	19.50^{b}±0.30	3.75^{e}±0.06	6.00±0.09	6.50^{b}±0.10	6.80^{de}±0.10	9.00^{e}±0.14	13^{d}±0.20	6.80^{de}±0.10	9.00^{e}±0.14	13^{d}±0.20
R.S - tn 4	10.95^{de}±0.17	14.10^{c}±0.21	17.50^{c}±0.27	3.75^{e}±0.06	6.10^{b}±0.09	5.50^{d}±0.09	7.20^{bc}±0.11	8.00^{f}±0.12	12^{e}±0.18	7.20^{bc}±0.11	8.00^{f}±0.12	12^{e}±0.18
R.S - ka 1	11.00^{de}±0.16	14.20±0.22	20.00^{b}±0.31	4.20^{d}±0.06	5.20^{d}±0.08	6.00^{c}±0.09	6.80^{de}±0.10	9.00^{e}±0.14	14^{e}±0.21	6.80^{de}±0.10	9.00^{e}±0.14	14^{c}±0.21
R.S - ka 2	11.10^{cd}±0.15	15.30^{b}±0.24	19.50^{b}±0.30	4.60^{bc}±0.07	5.50^{bc}±0.09	6.50^{b}±0.10	6.50^{b}±0.10	9.80^{cd}±0.15	13^{d}±0.20	6.50^{b}±0.10	9.80^{cd}±0.15	13^{d}±0.20
R.S - ka 3	10.35^{f}±0.16	14.20^{c}±0.22	21.50^{a}±0.33	3.35^{f}±0.05	5.30^{cd}±0.08	6.50^{b}±0.10	7.00^{cd}±0.11	8.90^{e}±0.14	15^{b}±0.23	7.00^{cd}±0.11	8.90^{e}±0.14	15^{b}±0.23
R.S - ka 4	12.20^{a}±0.19	13.20^{d}±0.20	21.60^{a}±0.33	4.20^{d}±0.06	5.20^{d}±0.08	5.60^{d}±0.09	8.00^{b}±0.12	8.00^{f}±0.12	16^{a}±0.24	8.00^{b}±0.12	8.00^{f}±0.12	16^{a}±0.24
R.S - kl 1	11.70^{b}±0.18	12.10^{e}±0.18	21.50^{a}±0.33	4.50^{c}±0.07	5.10^{d}±0.08	6.50^{b}±0.10	7.20^{bc}±0.11	7.00^{g}±0.11	15^{b}±0.23	7.20^{bc}±0.11	7.00^{g}±0.11	15^{b}±0.23
R.S - kl 2	10.30^{f}±0.16	15.20±0.23	19.80^{b}±0.30	3.40^{f}±0.05	5.20^{d}±0.08	5.80^{cd}±0.09	6.90^{cd}±0.11	10.00^{c}±0.15	14^{e}±0.21	6.90^{cd}±0.11	10.00^{c}±0.15	14^{c}±0.21
Untreated (Control)	12.50^{a}±0.19	16.50±0.25	22.00^{a}±0.34	5.00^{a}±0.08	5.50^{bc}±0.09	7.00^{a}±0.11	7.50^{b}±0.12	11.00^{a}±0.17	15^{b}±0.23	7.50^{b}±0.12	11.00^{a}±0.17	15^{b}±0.23

Toppe – Coffee seedling stage, BF: Butter fly stage of coffee seedlings, 3m: 3-month old coffee seedlings

khun. Growth Agar plugs (6 mm in diameter) cut from 7-day old cultures of *Rhizoctonia solani* khun were placed each opposite to those of tested fungal antagonists. For bacterial antagonists, 10 µl of each bacterial cell suspension streaked in the Petri plates using a sterile inoculation needle. Control plates were challenged with pathogen plugs only and bacterial suspension was replaced by a same volume of Sterile Distilled Water.

All culture plates were incubated at ± 27 °C for 2 days. Three plates were used for each individual treatment and the whole experiment was repeated twice. The diameter of pathogen colony was measured and microscopic observations were made to characterize the hyphal pathogen-antagonist interactions.

$$L = [(C - T) / C] \times 100$$

Where L is the percentage inhibition of radial mycelial growth, C is radial growth of the pathogen in the control; T is radial growth of the pathogen in the presence of *Trichoderma* spp (Edington *et al*., 1971).

In vivo biocontrol trials

In order to evaluate the ability of fungal and bacterial agents tested to reduce damping-off and Rhizoctonia collar rot disease, three biocontrol assays were performed.

Assessment of pre-emergence damping-off suppression ability

Ten coffee seeds were soaked for 10 – 15 min in each antagonist suspension prepared as previously described and sown in polybag filled with sterilized pot mixture mixed with an aggressive *Rhizoctonia solani khun.* Isolate (R.s- tn 4) at the rate of 5%. Poly bags were then kept at net house condition.

Pre-emergence damping-off percentage was recorded after 15 days of incubation based on the number of non-emerged seeds in relation to the number of total sown seeds.

Assessment of post-emergence damping-off suppression ability

Coffee butterfly seedlings (60-day-old) grown in ploy bags were treated by root dipping for 30 min in the spore or cell suspension of each fungal or bacterial antagonist, respectively. Treated seedlings were transplanted in poly bags filled with pot mixture infected with an aggressive *Rhizoctonia solani* khun. Isolate (R.s-tn4) at the rate of 5%. Inoculated and uninoculated control plants were root dipped in SDW and transplanted in pathogen-inoculated and pathogen-free substrates, respectively. Ploy bags were incubated under net house conditions. Coffee seedlings were used per each individual treatment.

The parameters, recorded 7 days after post-transplanting were plant height, plant fresh weight, percentage of post-emergence damping-off and disease severity. Post-emergence damping-off (%) was based on the number of plants showing disease symptoms in relation to the total number of emerged seedlings while disease severity was estimated based on the density of *R. solani* lesions formed on collar rot according to the 0-5 scale detailed above.

Assessment of rhizoctonia collar rot suppression ability

Coffee seedlings (45-day-old) were antagonist-treated and transplanted in pathogen-infected or not substrate, as previously described pot mixture poly bags. For each antagonistic treatment, ten treated plants were separately placed in 8x5 size of polybag containing pot mixture poly bags containing (6:2:1) a mixture of jungle soil, Farm Yard Manure and sand substrate being infected with an aggressive *Rhizoctonia solani* khun isolate (R.s-tn 4). Untreated and inoculated seedlings were included in the assay. All the seedlings were incubated under the same net house conditions. Disease Incidence and plant growth parameters (plant height and shoot length and root length) were recorded 60, 75, 90 days' post transplanting.

Statistical analysis

The results were subjected to Randomized Block Design analysis of variance and means separations were carried out using the Student-Newman-Keuls (SNK) test at $P \leq 0.05$. ANOVA was performed using SPSS version 22.0.

Experiments were conducted according to a completely randomized design for *in vitro* and in *vivo* (3 replications and 11 treatments) in nursery trials.

Results

Comparative pathogenicity of *Rhizoctonia solani* isolates

Results given in **Fig. 1** showed that all tested *Rhizoctonia solani khun.* Isolates were pathogenic to coffee

seeds and induced variable preemergence damping-off depending on isolates as compared to the uninoculated control. R.S – tn 4, R.S – ka 3, R.S – kl 2 isolates were found to be the most aggressive ones by inducing complete inhibition of seed germination after two weeks after incubation (Fig. 1). However, the remaining isolates reduced seed germination by 40 to 80% over control.

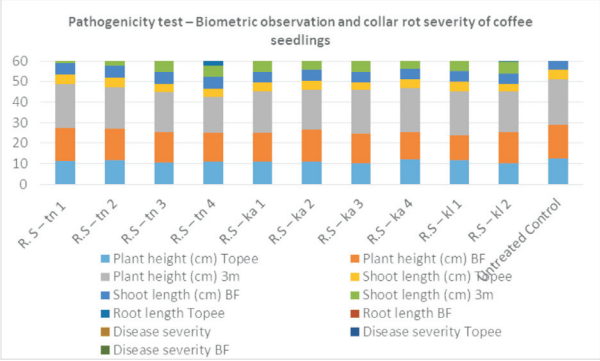

Fig. 1. Pathogenicity test – Biometric observation and collar rot severity of coffee seedlings

Biocontrol of *Rhizoctonia solani* by fungal and bacterial agents

In vitro antifungal activity of fungal antagonists

Dual culture technique

Selected four *Trichoderma* spp evaluated against *Rhizoctonia solani* khun by the dual culture technique (Morton and Strouble 1955). Mycelial discs 5 mm in diameter were excised from the edge of an actively growing antagonist and the pathogen was cultured an opposite ends of a petri dish equidistant from the periphery. A completely randomized experimental design was used with nine replicates for each isolate. In control petri dish, in place of antagonist, a sterile agar disc was inoculated on the side opposite to pathogen. Inoculated plates were incubated at 25 ± 10^0 C for 5- 7 days. After the incubation period, radial growth of pathogens was measured and the percent inhibition of average radial growth was calculated relative to the controls as follows:

$$L = [(C – T) / C] \times 100$$

Where L is the percentage inhibition of radial mycelial growth, C is radial growth of the pathogen in the control T is radial growth of the pathogen in the presence of *Trichoderma* spp (Edington *et al*., 1971).

The degree of antagonism between each of the *Bacillus subtilis (coffee native)* 79%, *Bacillus subtilis (76%), Trichoderma harzianum (coffee native) 75%, Trichoderma harzianum (72%)* and followed *by* Carbendazim 0.02% to compare to control (Bell *et al*., 1982).

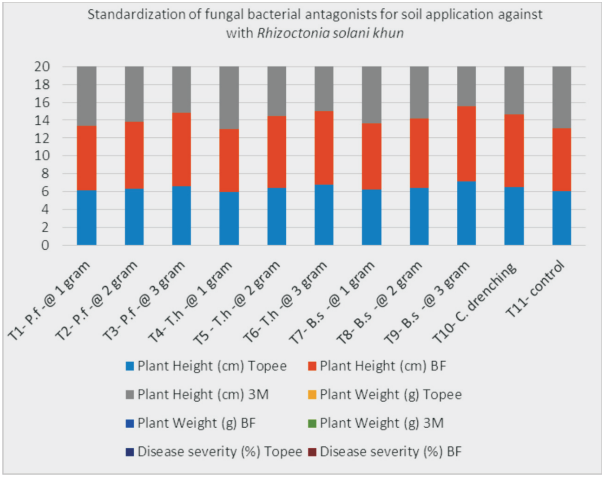

Fig. 2. Standardization of biocapsules antagonists for soil application with *Rhizoctonia solani khun*

Table 3. Performance of coffee seedling height with coffee bio-capsules

Treatment	Plant Height (cm)		
	Topee	BF	3M
T_1- P.f-@ 1 gram of biocapsule	6.20^{cde} ± 0.13	7.20^{de} ± 0.15	14^{fg} ± 0.29
T_2- P.f-@ 2 gram of biocapsule	6.35^{bcde} ± 0.13	7.50^{cd} ± 0.16	14.3^{efg} ± 0.30
T_3- P.f-@ 3 gram of biocapsule	6.64^{bc} ± 0.14	8.15^{ab} ± 0.17	16^{abc} ± 0.33
T_4- T.h-@ 1 gram of biocapsule	6.00^{e} ± 0.13	7.00^{e} ± 0.15	13.8^{fg} ± 0.29
T_5- T.h-@ 2 gram of biocapsule	6.45^{bcde} ± 0.13	8.00^{ab} ± 0.17	15.2^{cde} ± 0.32
T_6- T.h-@ 3 gram of biocapsule	6.79^{ab} ± 0.14	8.25^{ab} ± 0.17	16.503^{ab} ± 0.34
T_7- B.s-@ 1 gram of biocapsule	6.25^{cde} ± 0.13	7.40^{cde} ± 0.15	14.2^{efg} ± 0.30
T_8- B.s-@ 2 gram of biocapsule	6.40^{bcde} ± 0.13	7.80^{bc} ± 0.16	14.8^{def} ± 0.31
T_9- B.s-@ 3 gram of biocapsule	7.14^{a} ± 0.14	8.43^{a} ± 0.18	17.01^{a} ± 0.36
T_{10}- Carbendazim 3 gram	6.50^{bcd} ± 0.13	8.10^{ab} ± 0.17	15.503^{bcd} ± 0.32
T_{11}- Control	6.10^{de} ± 0.13	7.00^{e} ± 0.15	13.503^{g} ± 0.28

Statistical analysis

The analysis of variance was carried out and comparison was done by Duncan's Multiple Range Test (DMRT). The mean difference is significant at the P-values < 0.05. Statistical analysis was performed using the SPSS 16.0 software (SPSS Inc., Chicago, USA). Data presented are means from three replicates with standard errors. Within each treatment, different letters at each column indicate significant

Table 4. Performance of coffee seedlings weight against coffee bio-capsules

Treatment	Plant Weight (gram)		
	Topee	BF	3M
T_1- P.f-@ 1 gram of biocapsule	$1.80^{g} \pm 0.04$	$5.00^{fg} \pm 0.10$	$10.50^{e} \pm 0.22$
T_2- P.f-@ 2 gram of biocapsule	$2.10^{ef} \pm 0.04$	$5.20^{ef} \pm 0.11$	$11.50^{d} \pm 0.24$
T_3- P.f-@ 3 gram of biocapsule	$2.70^{c} \pm 0.06$	$6.00^{bc} \pm 0.13$	$12.60^{abc} \pm 0.26$
T_4- T.h-@ 1 gram of biocapsule	$1.70^{gh} \pm 0.04$	$4.80^{g} \pm 0.10$	$10.20^{e} \pm 0.21$
T_5- T.h-@ 2 gram of biocapsule	$2.50^{d} \pm 0.05$	$5.50^{de} \pm 0.11$	$12.20^{cd} \pm 0.26$
T_6- T.h-@ 3 gram of biocapsule	$3.00^{b} \pm 0.06$	$6.20^{b} \pm 0.13$	$13.00^{ab} \pm 0.27$
T_7- B.s-@ 1 gram of biocapsule	$2.00^{f} \pm 0.04$	$5.10^{fg} \pm 0.10$	$10.75^{e} \pm 0.22$
T_8- B.s-@ 2 gram of biocapsule	$2.20^{e} \pm 0.05$	$5.30^{ef} \pm 0.11$	$12.00^{cd} \pm 0.25$
T_9- B.s-@ 3 gram of biocapsule	$3.50^{a} \pm 0.07$	$7.00^{a} \pm 0.15$	$13.20^{a} \pm 0.28$
T_{10}- Carbendazim 3 gram	$2.60^{cd} \pm 0.05$	$5.70^{cd} \pm 0.12$	$12.30^{bc} \pm 0.25$
T_{11}- Control	$1.60^{h} \pm 0.03$	$1.60^{h} \pm 0.03$	$4.60^{f} \pm 0.10$

Table 5. Coffee collar rot incidence against coffee bio-capsules

Treatment	Disease incidence (%)		
	Topee	BF	3M
T_1- P.f-@ 1 gram of bio capsule	$8.25^{c} \pm 0.25$	$7.25^{c} \pm 0.25$	$6.25^{c} \pm 0.25$
T_2- P.f-@ 2 gram of bio capsule	$6.75^{e} \pm 0.75$	$5.11^{d} \pm 0.11$	$6.20^{c} \pm 0.20$
T_3- P.f-@ 3 gram of bio capsule	$5.80^{f} \pm 0.80$	$4.20^{e} \pm 0.20$	$3.12^{d} \pm 0.12$
T_4- T.h-@ 1 gram of bio capsule	$10.20^{b} \pm 0.20$	$8.11^{b} \pm 0.11$	$10.20^{b} \pm 0.20$
T_5- T.h-@ 2 gram of bio capsule	$8.20^{c} \pm 0.20$	$7.20^{c} \pm 0.20$	$6.24^{c} \pm 0.24$
T_6- T.h-@ 3 gram of bio capsule	$2.77^{g} \pm 0.77$	$3.20^{f} \pm 0.20$	$1.20^{e} \pm 0.20$
T_7- B.s-@ 1 gram of bio capsule	$7.12^{d} \pm 0.12$	$5.12^{d} \pm 0.12$	$6.20^{c} \pm 0.20$
T_8- B.s-@ 2 gram of bio capsule	$6.12^{e} \pm 0.12$	$5.12^{d} \pm 0.12$	$6.24^{c} \pm 0.24$
T_9- B.s-@ 3 gram of bio capsule	$1.20^{h} \pm 0.20$	$1.02^{g} \pm 0.02$	$1.2^{f} \pm 0.02$
T_{10}- Carbendazim 3 gram	$1.02^{i} \pm 0.02$	$1.03^{h} \pm 0.03$	$1.3^{f} \pm 0.03$
T_{11}- Control	$13.20^{a} \pm 0.20$	$15.75^{a} \pm 0.75$	$16.20^{a} \pm 0.20$

Treatment details

T_1- *Pseudomonas fluorescence* as Soil application @ 1 gram of capsule	T_4- *Trichoderma harizianum* as Soil application @ 1 gram of capsule	T_7- *Bacillius subtilis* as Soil application @ 1 gram of capsule	T_{10}- *Carbendazim* 3 gram of capsule
T_2- *Pseudomonas fluorescence* as Soil application @ 2 gram of capsule	T_5- *Trichoderma harizianum* as Soil application @ 2 gram of capsule	T_8- *Bacillius subtilis* as Soil application @ 2 gram of capsule	T_{11}-Control 3
T_3- *Pseudomonas fluorescence* as Soil application @ 3 gram of capsule	T_6- *Trichoderma harizianum* as Soil application @ gram of capsule	T_9- *Bacillius subtilis* as Soil application @ 3 gram of capsule	

Topee – Coffee seedling stage, BF: Butter fly stage of coffee seedlings, 3m: 3 month old coffee seedlings

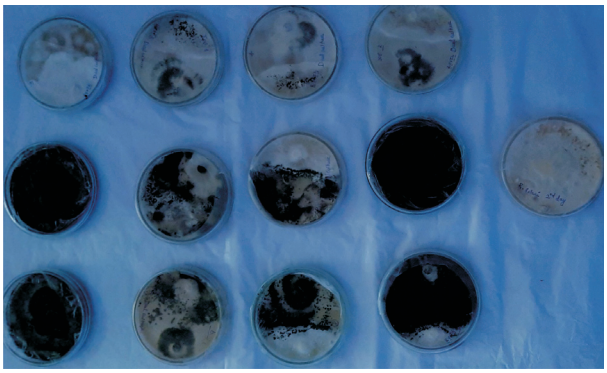

Plate 9. *Trichoderma harzianum* antagonists effect on 7th day

Pre emergence

Post emergence

differences by Duncan's multiple range test at P<0.05.

Results and Discussion

The present study investigates the pathogenicity / aggressiveness of different *Rhizoctonia solani khun.* Isolates issued from coffee seed towards one cultivar of Chandragiri and Rs. Tn 4 isolate caused pre-emergence and post-emergence damping-off (collar rot). These findings are also in agreement with previous studies reporting the pathogenicity of different isolates of *Rhizoctonia solani khun.* Isolated from root / hypocotyl of rotted coffee seedlings and found that all isolates were pathogenic and caused collar rot, pre-emergence, post-emergence damping-off coffee seedlings (Mahmoud *et al.*, 2007; Rahad *et al.*, 2012).

Table 6. *In vitro* screening of *Bacillus subtilis, Trichoderma harzianum, Pseudomonas fluorescens* against *R. solani Kuhn*

Treatment	Mycelia inhibition of *R. solani* (%)
Bio control agents	54.98^{d}±0.840
Trichoderma harzianum	55.10^{d}±0.840
Trichoderma harzianum coffee native	54.52^{d}±0.835
Pseudomonas fluorescens	54.60^{d}±0.835
Pseudomonas fluorescens coffee native	56.15^{cd}±0.855
Bacillus subtilis	58.65^{bc}±0.896
Bacillus subtilis coffee native	59.10^{b}±0.901
Carbendazim 0.02%	70.50^{a}±1.078

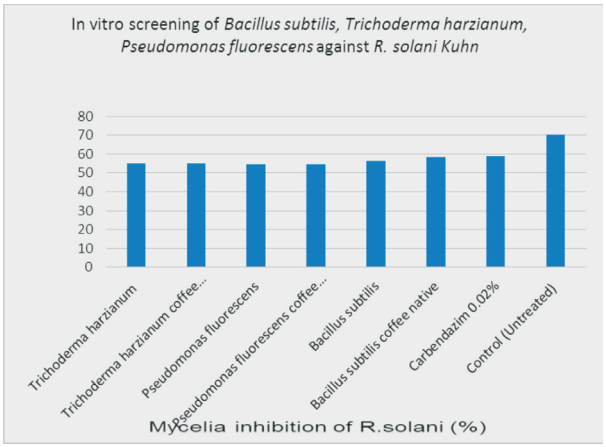

Fig. 3. *In vitro* screening of *Bacillus subtilis, Trichoderma harzianum, Pseudomonas fluorescens* against *R. solani Kuhn*

The biocontrol ability of three one fungal antagonists and two bacterial antagonists (*T. harzianum*, *Pseudomonas fluorescens and Bacillus subtilis)* against *Rhizoctonia solani* khun was also studied. In fact, species of the genus *Trichoderma harzianum* are the most widely used antagonists for controlling plant diseases caused by fungi due to their ubiquitous nature, ease with which they can be isolated and cultured, their rapid growth on a variety of substrates (Papavizas *et al.*, 1982). The mechanisms by which *Trichoderma* spp. suppress phytopathogens are basically three, *i.e.* direct competition for space or nutrients (Elad *et al.*, 1985), the production of antibiotic

metabolites, whether volatile or not (Chet *et al.,* 1997; Sid Ahmed *et al.,* 2000) and direct parasitism on phytopathogenic fungi (Yediai *et al.,* 1999). Furthermore, the genus *Trichoderma* possesses good qualities for controlling diseases in plants caused by soil borne pathogens, especially those of the genera *Phytophthora*, *Rhizoctonia* and *Macrophomina* (Sathiyaseelan *et al.,* 2009, Howell, 2003), *Pythium* (Benhamou *et al.,* 1997; Daami-Remadi M. 2001), *Fusarium* (Sathiyaseelan *et al*., 2009, Hibar K *et al*., 2005; Sahi *et al*., 2007).

Results from our study indicated that *Rhizoctonia solani khun.* mycelial growth was slightly inhibited by the antagonists tested. However, microscopic observations at the confrontation zone between *Bacillus subtilis* or *Trichoderma harzianum* and *Rhizoctonia solani khun.* Showed a profound change in the pathogen's mycelium: lysis, formation of mycelium cords and a coiling of antagonist's mycelium around pathogen; reflecting the mycoparasitism mechanism deployed by these antagonists. Similar effects were induced on *F. oxysporum* f. sp. *tuberosi* by the same antagonists tested in the present study (Ayed *et al*., 2006). Additionally, *T. harzianum* used against *F. solani* var. *coeruleum*, *F. roseum* var. *sambucinum* and *F. roseum* var. *graminearum* also caused a significant mycelium lysis (Daami-Remadi, 2001). An alteration of the mycelium of *Sclerotium rolfsii* was also induced by *T. harzianum* (Benhamou *et al*., 1996). Our results are consistent with those of Howell (Howell, 2003) who demonstrated that *T. lignorum* is able to wrap around the mycelium of *Rhizoctonia solani khun.* Causing dissolution of the pathogen's cytoplasm. Similar mechanisms (mycoparasitism and lysis) were deployed by *T. harzianum*, *T. viride* and *T. aureoviride* during them *in vitro* interaction with *R. solani* (Shalini *et al*., 2007). In addition, many studies have shown that *Trichoderma* species are capable to produce extracellular lytic enzymes (Elad *et al*., 1982).

The current study clearly demonstrated that all treatments tested for the control of collar rot of coffee seedlings the performed using the fungal antagonists and bacterial antagonists had significantly increased plant growth. Indeed, treatment with *Bacillus subtilis* or *Trichoderma harzianum* increased plant height by 7.14 cm and 6.79 cm at topee stage of seedling compared to *Rhizoctonia solani* khun. inoculated control in pot respectively. Similarly, treatment of coffee seedlings with *Bacillus subtilis*, *Trichoderma harzianum, Pseudomonas fluorescens,* Carbendazim 0.02% led to an increase by more than 50% of their root and aerial parts fresh weights compared to *R. solani*-inoculated and untreated control (Jabnoun-Khiareddine *et al*., 2009).

A reduction in disease severity antagonists effect on coffee collar rot was also obtained using *Bacillus subtilis*, *Trichoderma harzianum, Pseudomonas fluorescens,* Carbendazim 0.02% based-treatments. This reduction reached 58.65% with *Bacillus subtilis*, 55.10% with *T. harzianum* and 54.52% with *Pseudomonas fluorescens*, relative to *Rhizoctonia solani khun.* inoculated and untreated control.

In the present work, also noted a decrease in coffee collar rot incidence on coffee seedlings treated with the three tested antagonistic fungi bacteria by more than 50% compared to *Rhizoctonia solani* khun. inoculated and untreated control. These findings confirm those of Rini and Sulochana 2006 who showed that *Trichoderma harzianum* is more effective than *P. fluorescens* and *T. pseudokoningii* in controlling *Rhizoctonia solani khun.* In greenhouse and field grown pepper where root rot was reduced by 22.9%. Other previous studies also reported differences in the antagonistic potential of *Trichoderma* species isolated from a suppressive soil and shown active against *V. dahliae* (Berg. G *et al*., 2001, Berg. G *et al*., 2005). The *in vitro* evaluation of tree fungal bacterial isolates for the control of *Rhizoctonia solani* khun. showed that *Bacillus subtilis* and *Trichoderma harzianum* are the most effective.

The present study showed that *Bacillus subtilis* based treatment increased coffee seedlings fresh weight by 7 grams in butterfly stage, 3.5 g of topee seedlings. The *Trichoderma harzianum* 3 g in topee stage and 6.2 g in butterfly stage of seedlings and height 7.14 cm and 6.79 cm as recorded topee stage (40 days) and butterfly stage (60 days) (De Curtis *et al*., 2010). In the present study, *Bacillus subtilis*, *Trichoderma harzianum and Pseudomonas fluorescens* suppressed by 58.65%, 55.10% and 54.60% mycelial growth inhibition over control against *Rhizoctonia solani respectively*.

In the preliminary investigation, an attempt was made to screen the antagonistic potential of coffee rhizosphere native bio control agents against *Rhizoctonia solani khun.* The causal organism of coffee collar rot disease in coffee seedlings. The isolated pathogen was identified as *Rhizoctonia solani khun.* (R.s Tn 4) based on their colony morphology by using standard fungal manuals (Pathak 1987). The present study revealed that *invitro* dual culture tech-

nique, the isolated and identified selected three biocontrol fungal agents are *Bacillus subtilis, Trichoderma harzianum, Pseudomonas fluorescense and* Carbendazim (0.02%) were tested the efficiency of inhibiting the mycelial growth of *Rhizoctonia solani khun.* Among the three bio control agents the highest antagonistic effect against the mycelial growth inhibition of pathogen was found with *Bacillus subtilis (coffee native)* 79%, *Bacillus subtilis* (76%), *Trichoderma harzianum (coffee native)* 75% and followed *by* Carbendazim 0.02% to compare to control respectively Table: 1. and Plate.3, 4,5.

Identified efficient strains *Trichoderma harzianum and P. flouroscens, B. Subtilis* had 91.0 per cent of inhibition (Soundara Rajan *et al.*, 2020). Although, Wilson *et al*. (2008) found that genus *Trichoderma*, species such as *Trichoderma. hamatum*, *Trichoderma. harzianum, Trichoderma reesei*, *Trichoderma virens*, and *Trichoderma viride* have demonstrated excellent antagonistic activity against *Rhizoctonia solani* on potato pot and or field tests. Shafiquzzaman *et al*. (2009) reported that *Trichoderma harzianum* (Th), *Trichoderma. viride* (Tv) and *Trichoderma. koningii* (Tk) upon evaluation via dual culture technique resulted in suppression of soil borne pathogens of different vegetables viz. *Rhizoctonia solani*, *Sclerotium rolfsii* and *Sclerotinia sclerotiarum* under *in vitro* conditions. Similarly, *Trichoderma. viride* (Tv-1), *Trichoderma. viride* (Tv-2), *Trichoderma. harzianum* (Th-1) inhibited the growth of *Rhizoctonia solani* Radwan *et al*. (2007). *Trichoderma virens* $T_5$23 and *Trichoderma* sp. were evaluated against five isolates of soil borne phytopathogenic fungi via dual culture techniques resulted in suppression of *Fusarium graminearum, R. solani (AG4 and AG5), Macrophomina phaseoli* and *Phytophtora cacturum* Behzad *et al*., (2008).

Conclusion

In the present study, all *Rhizoctonia solani khun.* isolates tested were shown to be pathogenic to coffee plants with variable degree of aggressiveness noted on the one cultivar of Chandragiri. These isolates were able to induce pre- and post-emergence damping-off and collar rot coffee disease as well as affect the plant growth reduction.

In an attempt to biologically control this disease, fungal and bacterial isolates were tested *in vitro* and *in vivo* against *Rhizoctonia solani khun.* Our results demonstrated that some of the tested biocontrol agents, applied at different coffee seedlings growth stages were able to suppress disease and to improve plant growth. Their effectiveness will be further evaluated under field conditions.

The result indicated that collar rot incidence was less in T_{10} - *Carbendazim* 3 g of capsule and T_9- *Bacillus subtilis* -@ 3 g of bio-capsule treatment followed by *T6 - Trichoderma harizianum* @ 3 g of bio-capsule were observed and significantly in their experiment in collar rot of coffee. The present investigation amply proved the reducing of collar rot was managed by alternative chemical way of coffee bio-capsule of *Bacillus subtilis* and *Trichoderma harizianum*. Since coffee is a transplanting crop, growers can easily follow inoculum efficient culture of *Bacillus subtilis* in coffee nursery. This is simple low-cost and novel technology will play an important role in getting healthy and vigours coffee seedlings by coffee biocapsule method.

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