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Synergistic effect of biocontrol agents and chitosan on control of foot rot disease in finger millet

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Abstract
Finger Millet (*Eleusine coracana* L. Gaertn.) is a widely grown millet crop in Southern Karnataka, it is attacked by various fungal, bacterial and virus pathogens, among them foot rot disease caused by *Sclerotium rolfsii* is a leading constraint for the farmers under both irrigated and rainfed conditions. For the management of this problem an eco-friendly approach was taken up in laboratory condition using chitosan and biocontrol agents, as they are known to have spin-off benefit of active innate defence mechanisms and promoting higher yields. In the research, chitosan of nine different concentrations were selected among them 0.15%, 0.20% and 0.25% showed the maximum inhibition of the pathogen. Along with chitosan, bio-agents *viz*., *Trichoderma harzianum* and *Pseudomonas fluorescens* were evaluated against *Sclerotium rolfsii* for their synergistic effect by well diffusion technique and it exhibited a positive effect on the inhibition of the pathogen showing the effectiveness in reducing the disease and chitosan not showing deleterious effect on bio-agents.

Keywords: Chitosan, foot rot, biocontrol agents (*Pseudomonas fluorescens* and *Trichoderma harzianum*) and synergistic effect

Introduction
The rapid growth of global population and the globalization of the economy are constantly putting pressure on the agriculture sector. Agriculture is struggling to support the present scenario, added to this problem, plant diseases curtails the production and quality of crop. India being a tropical country where environmental conditions are conducive for the growth of the pathogens, which distresses crop growth, quality and yield.

In the wake of the green revolution, crop protection relied heavily on chemical fertilizers and pesticides. However, the chemicals are a double-blade sword which will help in increasing productivity but on the other hand it is harming biological diversity as toxins enters the food chain and development of resistance strains (Sun *et al*., 2012) [31]. So, to escape from this immense threat we need to adopt sustainable measures for controlling the plant diseases without deteriorating the quality and safety of agricultural products and commodities by following environmentally friendly approaches. Biological control of plant pathogens can reduce crop losses, assures food security, and helps low-income farmers.

Finger Millet (*Eleusine coracana* L. Gaertn.) belongs to the family Poaceae. With India being leading producer, Finger millet is cultivated mainly in Asia and African countries like India, Japan, China, Srilanka, Malaysia, parts of Central and Eastern Africa. Finger millet is widely grown as annual cereal herbaceous crop in the arid and semiarid areas and known as *bird's foot*, *madua*, *mandua*, *maruva*, *nachni*, *nagli*, *ragi etc*., in various parts of the country, it is mainly concentrated in Southern India. In India, Finger millet was grown under area of 1.19 m ha^{-1} with production and productivity of 1.98 mt and 1662 kg ha^{-1}, respectively (India Agristat, 2017-18)

In Southern part of India, ragi is used for food, ragi straw is used to feed cattle, the malted grains can be used as nutritious food for new-borns and is recommended patients suffering from diabetes. Now a days processed ragi products are also consumed such as biscuits, cookies, pasta and vermicelli, *etc*., Ragi is the cheapest source of nutrition compared to cereals

and most preferred food crop for physically hard working people. It provides fairly good amount of proteins, minerals, calcium, iron and vitamins in abundance. Protein present in finger millet, reported to possess a fairly high biological value, which is needed for the maintenance of nitrogen equilibrium of the body. Ragi contains high amount of fiber which helps to prevent stiffness, pain, cholesterol formation and intestinal cancer (Malleshi and Hadimani, 1993) [16]. Though ragi is familiar as hardiest crop, it is susceptible to many pathogenic diseases such as foot rot, blast, downy mildew, smut, blight, mosaic, and mottling mainly in high yielding varities (Govindu and Shivanandappa, 1967) [8].

Foot rot is caused by *Sclerotium rolfsii*, a prominent disease of ragi mainly in irrigated and high rainfall areas (Nagaraja and Reddy, 2009; Saccardo, 1913) [22, 29]. Since the use of chemical pesticides has to be reduced we have to go for an environmental friendly approach like exploring microbial inoculants and use of some organic products to manage and prevent the disease. Many species of bacteria and fungus are reported to be effective and act as antagonist against soil borne plant pathogens. Biological control agents act as viable and potent alternatives to conventional chemicals for the effective control of soil borne plant diseases (Jeyarajan and Angappan, 1998) [12]. Bacterial isolates such as *Pseudomonas fluorescens* open the gates for component of integrated disease Management for sustainable agriculture (Mukhopadhyay *et al*., 1987) [19]. The isolates of *Trichoderma harzianum* are efficient in suppressing *S. rolfsii* (Manu *et al*., 2012; Patro and Madhuri, 2013) [17].

Chitosan is derived from chitin as chitosan is eco-friendly, it is used in agriculture as a biocontrol agent for the plant protection against harmful phyto-pathogens (fungi, bacteria, viruses and abiotic stress) (Li *et al*., 2013). Mainly chitosan molecules are known for the induction of the host plant resistance (HPR). Treatment of the chitosan to the plant tissues strengthens natural defense mechanism and improves the physiological properties in the plant (Ghauoth *et al*., 1994) [7].

Since there are no literatures available to manage foot rot disease in ragi using bio-control agents and chitosan. The present study focused on Synergistic effect of bio-control agents and chitosan to control the foot rot disease.

Methodology

In vitro study on Synergistic effect of Biocontrol agents and Chitosan to control the Foot rot disease in Finger millet was conducted in the Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bengaluru during the year 2020.

Chitosan flakes used for the present investigation was procured from Hi Media Laboratories, India. Biocontrol agents *viz*., *Pseudomonas fluorescens* and *Trichoderma harzianum* pure cultures were procured from biofertilizer scheme, Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Banglore-560065. Pure culture of *Sclerotium rolfsii* pathogen causing foot rot was procured from All India Coordinated Research Project on Small Millets, ZARS, VC Farm, Mandya, Karnataka - 571405.

Preparation of chitosan solution

The stock solution of 1mg/ml of chitosan was prepared by dissolving purified chitosan in glacial acetic acid under continuous stirring in mechanical shaker. Later the pH was adjusted to 5.5-6.0 using 2N sodium hydroxide, dialysed for 12 h against cold distilled water and autoclaved. From the stock solutions, nine different concentrations (0.01%, 0.03%, 0.05%, 0.07%, 0.09%, 0.12%, 0.15%, 0.20% and 0.25%) of chitosan were prepared with distilled water (Chittenden and Singh, 2009) [3].

Antimicrobial Activity of Chitosan against *Sclerotium rolfsii*

The inhibitory effect of chitosan at different concentrations were tested *in vitro* against *Sclerotium rolfsii*. For which nine different concentrations (0.01%, 0.03%, 0.05%, 0.07%, 0.09%, 0.12%, 0.15%, 0.20% and 0.25%) of the chitosan were added to the molten Potato dextrose agar (PDA; potato: 200g, dextrose: 20g, agar: 20g for one litre distilled water) media prior to pouring of the media into the Petri dishes. Plate which was not amended with chitosan was named as control. The plates were then inoculated with the *S. rolfsii* (test fungi). The 5mm core of the fungal inoculum from an actively growing colony was transferred to the center of the plate and incubated at modified temperature of 36±1°C for four days in the incubator. Set of 3 replicates were prepared for each concentration/per treatment. Zone of inhibition was measured by using following formula (Laflamme *et al*., 2000) [14].

$$I = \frac{C - T}{C} \times 100$$

Where,
I = Inhibition of pathogen growth
C= Pathogen growth in control
T= Pathogen growth in treatment

Antagonistic effect of biocontrol agents (*Pseudomonas fluorescens* and *Trichoderma harzianum*) against *Sclerotium rolfsii*

In vitro evaluation was done for the biocontrol agents *Pseudomonas fluorescens* and *Trichoderma harzianum* through dual culture technique for their antagonistic effect against *S. rolfsii* (Lahlali *et al*., 2007) [13]. King's B medium (peptone: 20g; Glycerol: 15ml; K_2HPO_4:2.5g; $MgSO_4.7H_2O$: 6g; Agar: 20g) for one litre distilled water) and PDA 1:1 (Messaoud, 2013) [18] ratio was used for the study of bacterial antagonist and test fungus which were cultured to get fresh and active growth of bio agents. Whereas PDA was used for the study of fungal antagonist and test fungus.

Dual culture technique

Biocontrol agent's evaluation was done for their efficacy by dual culture technique. King's B medium and Potato dextrose agar (PDA) were used for culturing of both the bio-control agents and test fungus. Twenty millilitre of sterilized and cooled King's B medium and Potato dextrose agar (PDA) was poured into the sterile Petri plate and allowed for solidification. In the evaluation of bacterial antagonist, the bacterium *Pseudomonas fluorescens* was streaked onto the Petri plates at one end *S. rolfsii* was placed oppositely to the other end of Petri plate and incubated at modified temperature of 36±1 °C for four days and the inhibition zone was measured by measuring the clear distance between the test fungus margin of the antagonistic organism and 5mm *S. rolfsii* mycelial disc was placed just opposite on other side. Later on plates were incubated at modified temperature of 36±1°C for four days and the inhibition zone was measured by measuring the clear distance between the test fungus

margins of the antagonistic organism. The diameter of the pathogen colony in control plate was also recorded. Whereas in-case of fungal antagonist, 5mm disc of *T. harzianum* was placed at one side of PDA media and 5mm disc of *S. rolfsii* on the other side of Petri plate. The per cent inhibition of the pathogen's growth was calculated by the formula given by Vincent (1947) [33].

$$I = \frac{C - T}{C} \times 100$$

Where,
I = Inhibition of pathogen growth
C= Pathogen growth in control
T= Pathogen growth in treatment

Synergistic effect of chitosan and biocontrol agents (*Trichoderma harzianum* and *Pseudomonas fluorescens*) against *S. rolfsii*

Agar well diffusion method:

The method used for evaluation of synergistic effect of chitosan and biocontrol agents against *S. rolfsii* by agar well diffusion method, (Valgas *et al*., 2007) [32]. Nine different concentrations of the chitosan (0.01%, 0.03%, 0.05%, 0.07%, 0.09%, 0.12%, 0.15%, 0.20% and 0.25%) were added to the mixed potato dextrose agar media and King's B media in 1:1 ratio and poured into the Petri dishes, keeping one plate as control (without chitosan and biocontrol agents). The plates were allowed to solidify and three wells with a diameter of 8 mm were punched, a volume of 20–100 µL culture was added in all the wells, in one of the well the spore suspension of *Trichoderma harzianum*, in the second well the spore suspension of *S. rolfsii* and in the third well the bacterial suspension of *Pseudomonas fluorescens* were added. Later on plates were incubated at modified temperature 36±1°C for four days and observed for the growth. The zone of inhibition was measured daily, Vincent (1947) [33].

$$I = \frac{C - T}{C} \times 100$$

Where,
I = Inhibition of pathogen growth
C= Pathogen growth in control
T= Pathogen growth in treatment

Statistical analysis was carried out as per the procedures given by Panse and Sukhatme (1985) [23].

Results and discussion

Effect of different chitosan concentrations on inhibition of *S. rolfsii*

The efficacy of nine different chitosan concentrations were studied against the pathogen *S. rolfsii* under *in-vitro* conditions. The results of the experiment exhibited that at higher concentration of chitosan, mycelial growth was inhibited significantly. In experimental studies, the mean mycelial diameter was measured and zone of inhibition was calculated every day. In case of control (T1) there was no inhibition, among all the nine different concentrations of chitosan the mycelial growth was inhibited at the concentration of chitosan at T8, 0.15% (1500ppm) and above concentrations *i.e*., T9 and T10. The zone of inhibition was 94.44% in treatments T8 (0.15%), T9 (0.20%) and T10 (0.25%) (Figure 1, Fig 2).

The results were in accordance with Pongphen *et al*. (2007), found that the chitosan has controlled the growth of the mycelia and germination of the spore in *Colletotrichum gloeosporioides.* Similar observation was made by Roller and Covill (1999) [28] in *Mucor racemosus*, Prapagdee *et al*. (2007) [24] on *F. solani* f.sp. *glycine*. The findings were in line with Reglinski *et al*. (2010), Abd-Alla and Haggag (2010) [1], El Hassni *et al*. (2004) [5] and Munoz and Moret (2010). The pathogen, *S. rolfsii* was inhibited due to the anti-fungal properties of chitosan. Benhamou (1992) [2] stated that, among all the natural elicitors, chitosan has a greater potential as a biodegradable substance, further possessing anti-microbial and eliciting activities.

The results of the experiment exhibited that chitosan at the higher concentration inhibited mycelial growth of *S. rolfsii* significantly due to the presence of natural elicitor compounds, anti-microbial activities of chitosan which includes Electrostatic interactions, Interaction with cell membrane and cell wall component, Interaction with the charged phosphate groups of DNA/RNA, Chelation of metals, Deposition onto the microbial surface and induction of plant defence mechanisms (Li *et al.*, 2008; Rabea *et al.,* 2009) [15, 25]. Exceptionally there was restraint of the pathogen at the higher concentration due to the anti-fungal mycelium growth, sporulation, morphology and molecular organization of fungus and elicitation of pathogenesis-related proteins, defence-related enzymes and defence-related secondary metabolites accumulation of Chitosan.

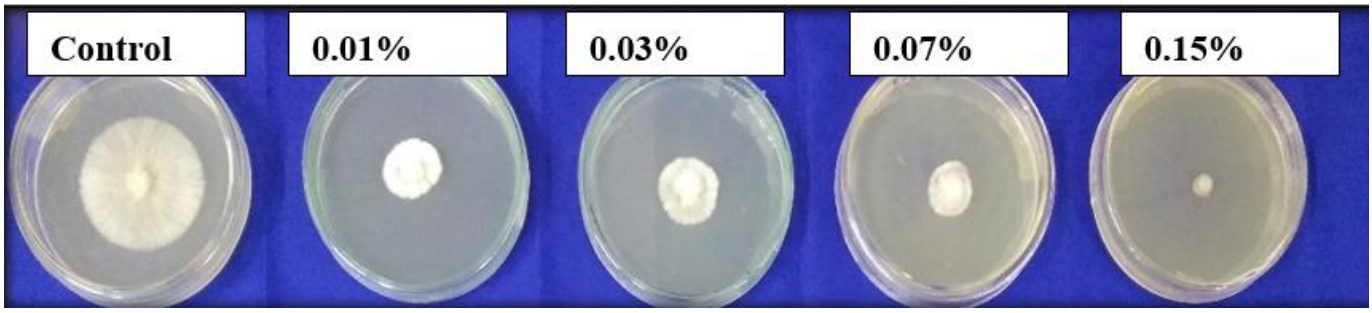

Fig 1: Effect of different concentrations of chitosan on inhibition of *S. rolfsii*

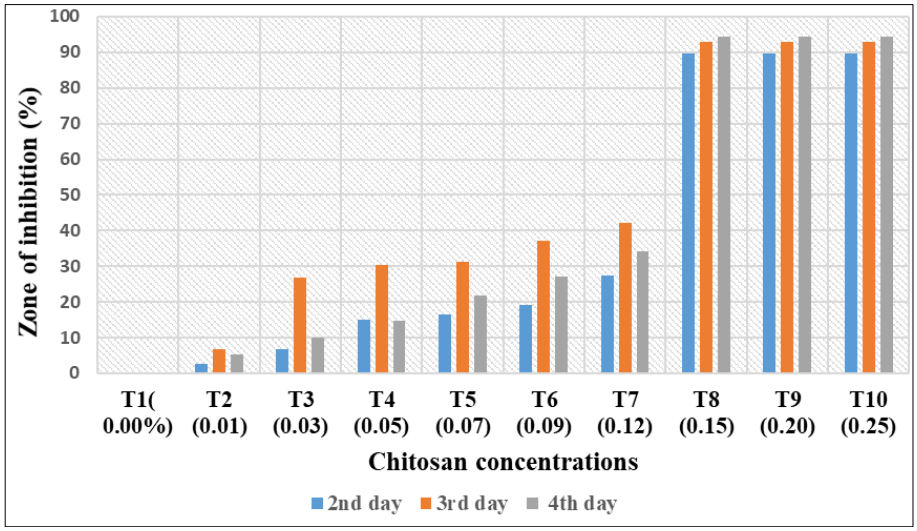

Fig 2: Effect of different chitosan concentrations on inhibition of *S. rolfsii*

Effect of bioagents (*Pseudomonas fluorescens* and *Trichoderma harzianum*) against *S. rolfsii*

Effect of *Pseudomonas fluorescens* against *S. rolfsii*

The antagonistic *Pseudomonas fluorescens* was evaluated against *S. rolfsii* by dual culture technique. The inhibition zone was measured in mm at 1st, 2nd and 3rd day after inoculation and the percent inhibition was calculated. The inhibition of the pathogen by *Pseudomonas fluorescens* was 10.97%, 30.55% and 45.69% at 1st, 2nd and 3rd day, respectively (Figure 3, Fig 4). The results were in accordance with Raveendra (2018) [27], the inhibition of *S.rolfsii* by different isolates of *Pseudomonas* ranged from 20.19% to 57.04%. These variations may be due to differential inhibitory efficacy of the isolates of *Pseudomonas*. Certain members of the *P. fluorescens* proved to be promising biocontol agents which inhibits plant diseases by safeguarding the seeds and roots from fungal infection. They are known to increase plant growth promotion and decrease acuteness of many fungal diseases (Hoffland *et al*. 1996, Wei *et al.* 1996) [11, 34]. It is because of the production of many secondary metabolites such as antibiotics, siderophores and hydrogen cyanide (O'Sullivan & O'Gara 1992) [21]. Hass and Defago (2005) [10] examined how the *P. fluorescens* control pathogenic microorganisms. Steijl *et al*. (1999) [30] working with radish and carnation for control of *F. oxysporum* f. sp. *Raphanin* and *F. oxysporum* f. sp. *Dianthi* found that fungal infection was inhibited and led to degradation of cell walls in host cells by *P. fluorescens*. The lignin component in infected wall was de methoxylated, oxidized and depolymerized. It was also found that infecting radish and carnation with *P. fluorescens* WCS 417r before pathogen infection led to reduced cell wall degradation. significantly there was inhibition of *S. rolfsii* by *P. fluorescens* due to the production of secondary metabolites against the pathogen.

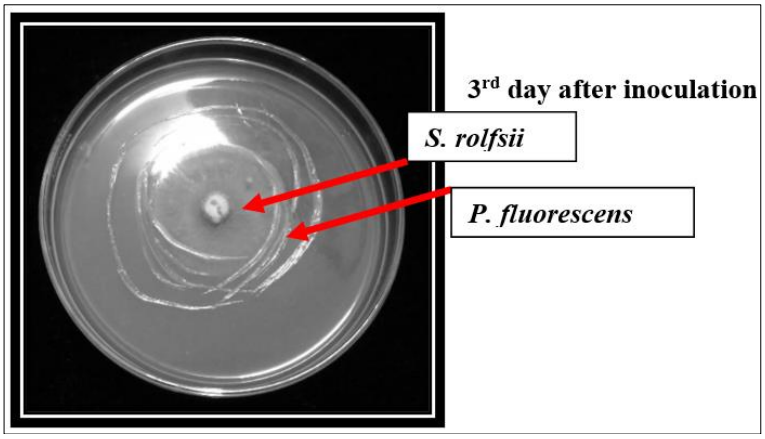

Fig 3: Effect of *Pseudomonas fluorescens* against *S. rolfsii*

Effect of *Trichoderma harzianum* against *S. rolfsii*

Trichoderma harzianum was evaluated against the pathogen, *S. rolfsii* using dual culture technique under *in vitro* conditions. The growth of the mycelia was studied everyday and the zone of inhibition was calculated. Remarkably there was quadrupling in the inhibition of pathogen's mycelia *i.e*., 15.66%, 35.47% and 52.55% at 1st, 2nd and 3rddays after inoculation, respectively (Figure 5, Fig 4). *In vitro* studies were conducted to evaluate antagonistic activities of *Trichoderma harzianum* against *S. rolfsii*. The inhibition of *S. rolfsii* was due to mycoparasitism, where *Trichoderma harzianum* suppressed the growth of *S. rolfsii*. Raveendra (2018) [27], studied on antagonistic activities of fungal bio-agent, *Trichoderma* spp. against *S. rolfsii* and found inhibition of mycelial growth ranged from 27.22 to 48.89 per cent. Various *Trichoderma* spp. are able to produce several plant defence eliciting microbe associated molecular patterns (MAMPs) such as xylanases, swollenins, peptaibols and cerato-platanins (Druzhinina *et al*., 2011; Harman *et al*., 2004) [4, 9]. Mycoparasitism is the methodology of suppression of *Sclerotium* by *Trichoderma* which assails and parasites the mycelia.

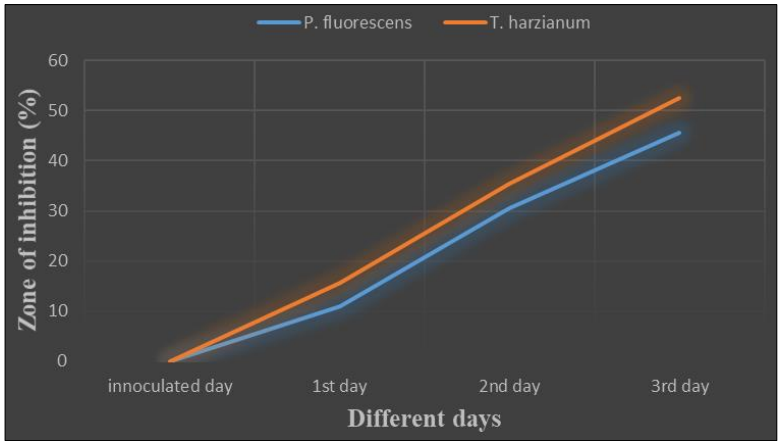

Fig 4: Effect of *Pseudomonas fluorescens* and *Trichoderma harzianum* against *S. rolfsii*

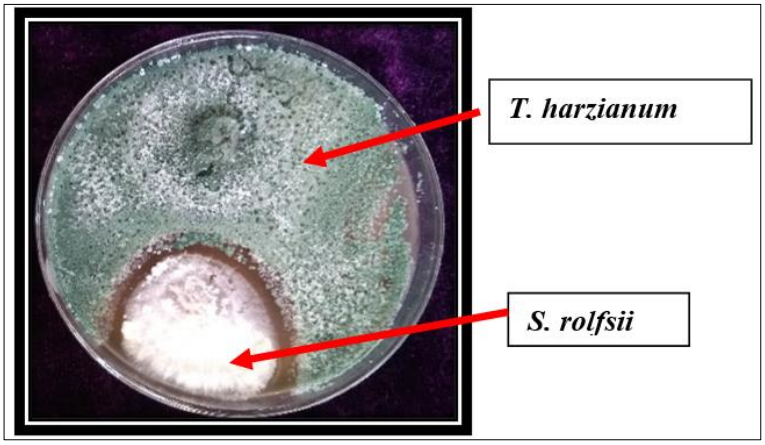

Fig 5: Effect of *Trichoderma harzianum* against *S. rolfsii.*

Synergistic effect of chitosan and biocontrol agents (*T. harzianum* and *P. fluorescens*) against pathogen using agar well diffusion method

In vitro studies of nine different chitosan concentrations and Bio-control agents (BCA) tested against *S. rolfsii* using Agar well diffusion method. The results revealed that after four days of incubation there was significant reduction in the per cent inhibition of mycelial growth of *S. rolfsii*. Among the combination of different chitosan concentrations and BCA, T8 (0.15%) and the above concentrations T9 (0.20%) and T10 (0.25%) of chitosan along with BCA was significantly superior over all the different concentrations along with bioagents by recording 94.44% inhibition of mycelial growth. In other concentrations, the inhibition of the mycelial growth ranged from 55.55% to 80.55% whereas, the control showed luxuriant growth without any inhibition. The inhibition of *S. rolfsii* may be due to mycoparasitism, production of secondary metabolites and anti-microbial activities of chitosan (Figure 6, Fig 7).

The results were in accordance with Ramakrisna (2017) [26], *in vitro* studies of the efficacy of two potential antagonists and chitosan against early blight of tomato showed maximum inhibition of *A. solani* by 53.44% @ 1000 ppm and 67.54% @ 1500 ppm. El-Mohamedy (2013) [6] reported that *T. harzianum*, *T. viride*, *Bacillus subtilis*, and *P. fluorescens* controlled tomato root rot pathogen under *in vitro* conditions. The inhibitor effect of the two tested chitosan (High and Low molecular weight) was increased as chitosan concentration is increased in growth medium to reach its maximum at the highest concentration (5 g/L). Complete inhibition of tested fungal growth was observed at the concentration of 4 g/L of LMW chitosan, while the highest fungal growth reduction was recorded in PDA-amended with HMW at the same concentration. The effect of chitosan at higher concentration and bio-control agents on the inhibition of the *S. rolfsii* was significant, chitosan and bio-control agents have the capacity to inhibit the mycelium and spores. Therefore they can be used for the further studies.

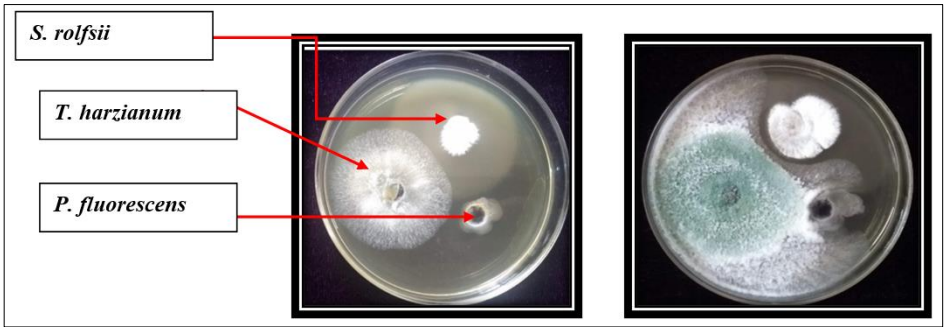

Fig 6: Synergistic effect of chitosan and *Trichoderma harzianum* on *S. rolfsii* under *in vitro* conditions

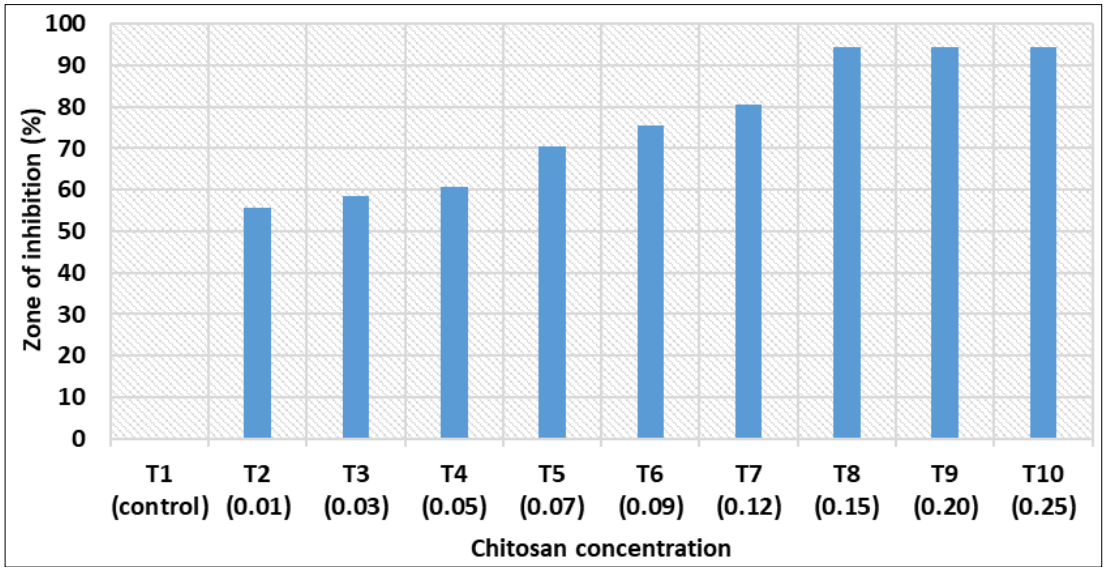

Fig 7: Synergistic effect of chitosan and bio-control agents (*Trichoderma harzianum* + *Pseudomonas fluorescens*) on *S. rolfsii*

Conclusion

The experimental results revealed the best concentration of chitosan among nine different chitosan concentrations against the pathogen *Sclerotium rolfsii*. The best three concentrations of chitosan (0.15%, 0.20% and 0.25%) inhibited the pathogen by 94.40%. The biocontrol agents were checked against *Sclerotium rolfsii*, the inhibition of *Sclerotium rolfsii* by *Pseudomonas fluorescens* was 45.69% whereas *Trichoderma harzianum* inhibited the pathogen by 52.55%. The synergistic effect of chitosan and bioagents showed 94.44% inhibition of *S. rolfsii* in all the three concentrations of the chitosan (0.15%, 0.20% and 0.25%). Though the results indicate that there is no much difference between using chitosan alone and in combination with biocontrol agents (0.04% more), but it has other advantages like increase plant growth, yield etc, when biocontrol agent is used, hence the chitosan (0.15%) and biocontrol agents can be used to control *S. rolfsii* as a eco-riendly method.

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