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Some *in vitro* Observations on the Biological Control of *Sclerotium rolfsii*, a Serious Pathogen of Various Agricultural Crop Plants

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Abstract: *The present study reveals the antagonistic potential of some fungi isolated from the agricultural field soil which were screened against the test pathogen (Sclerotium rolfsii) in vitro. Six fungal species viz. Penicillium sp, Aspergillus niger, Curvularia sp, Trichoderma harzianum, Trichoderma viride, and Fusarium sp were tested in vitro in dual culture by inoculating both the antagonist and the pathogen simultaneously (2cm apart). Among the six fungal isolates, Trichoderma harzianum and Trichoderma viride grew very fast compared to the pathogen (Sclerotium rolfsii) and inhibited its mycelium producing a clear inhibition zone. Maximum percentage of inhibition on the growth of the Sclerotium rolfsii was observed with Trichoderma harzianum (77.39%), followed by Trichoderma viride (76.54%) while considerable degree of inhibition was also observed with, Aspergillus niger (30.48%), Penicillium sp (29.05%) and Curvularia sp (13.57%). The study revealed the biocontrol potential of some naturally available soil fungi isolated from the agricultural field soil, which may be tried as biocontrol agents against Sclerotium rolfsii in the field condition.*

Keywords: *Antagonists, biological control, in vitro, pathogen, Sclerotium rolfsii, Trichoderma spp.*

I. Introduction

Sclerotium rolfsii is a well known polyphagous soil borne plant pathogenic fungus (Aycock, 1966), generally distributed in tropical and subtropical regions where soil temperature prevails around 30° (Harlapur, 1988). It was first observed in the United States by Peter Henry Rolfs (1892) as a cause of tomato blight in Florida. It is a serious pathogen on many crops of economic importance in most of the tropical and subtropical regions of the world. In India Shaw and Ajrekar (1915) isolated this organism from the rotting potatoes and Delphinium and it was identified as *Rhizoctonia destruens* tassi. In a later study by Ramakrishnan (1930), the fungus involved was identified as *Sclerotium rolfsii*. Sclerotium wilt or rot is a disease of tropics and subtropics. It has been reported that in most of the northern European countries the fungus do not survive in the soil for a long period of time because these regions are apparently too cold for this organism to survive. The fungus survives in soil mainly as sclerotia, which represent the main source of inoculum and it remains viable in soil for several months (Higgins, 1927). Most of the infection often extends from the stem into the roots of various hosts, but primary entry through the root system is much less common. Any other plant parts in contact with soil or near the surface are frequently infected. Maturing and ripen fruits are more susceptible. In fruits touching the soil surface, infection is usually initiates on the side in contact with the soil (Rosen and Shaw, 1929). Most of the first symptom associated with *Sclerotium rolfsii* are usually yellowing and wilting of leaves following stem rot infections.

Due to the adverse effect of chemical control of pest and diseases the attention for biological control is now increased by using some beneficial microorganisms (Erkol *et al*, 2011). Biological control is the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active or dormant state by using one or more microorganisms, accomplished naturally or through the manipulation of the environment, host or antagonist or by mass introduction of one or more antagonists (Baker and Cook, 1974).

Biological management of the disease through antagonists is a eco-friendly approach apart from better alternative to the use of chemicals. Among the soil microorganisms, there are forms that inhibit the growth of other microbes; these are called antagonists (Campbell, 1989; Morang *et al*., 2013). *Trichoderma sp*. have been reported to be potential antagonists and these gained considerable success for the control of plant diseases (Dennis and Webster, 1971; Dutta, 1981; Upadhya and Mukhopadhya, 1986). Wells *et al*. (1972) reported that *Trichoderma harzianum* as a biological amendment to field soil reduced the disease caused by *Sclerotium rolfsii.*

The idea of a sustainable agricultural practice and environmental protection is enhancing the need of biocontrol as an alternative technique to avoid chemical hazards on both human beings and beneficial soil microorganisms (Campbell, 1989). The application of biocontrol agents is the key elements for sustainable

agriculture. Therefore, the adoption of a sustainable agricultural practice, using strategies that are environmentally friendly, less dependent on agricultural chemicals is gaining worldwide recognition. In view of the above findings the present study was carried out *in vitro* by isolating some of the beneficial soil microorganisms from the agricultural field soil and tested against the *Sclerotium rolfsii* to determine their antagonistic potential *in vitro*.

II. Materials And Methods

Media used:

Potato dextrose agar (PDA) (Riker and Riker, 1936) medium was used for subculturing the test fungus, *Sclerotium rolfsii* and antagonism study was done under *in vitro* condition. Rose Bengal agar media (Tsao, 1964) was used for the isolation of fungi from the agricultural field soil.

Isolation and identification of the pathogen:

Diseased brinjal plant materials were collected from the agricultural field of Barak Valley, Assam and small pieces were made from the infected portion of the stem near the root zone. These were then sterilized with mercuric chloride (0.2%) (unsaturated solution in ethyl alcohol) or sodium hyphochloride (1%). It is then aseptically transferred to sterile petriplates containing sterile Potato dextrose agar media. It is later incubated at $28^0 \pm 2^0$ C for 3-5 days. The pathogen was identified based on the mycelia and sclerotia formation with the help of the standard mycological literature (Aycock, 1966; Barnett and Hunter, 1972). And the same was sub cultured in the sterile PDA slants for further studies.

Isolation and identification of the culturable fungi from the agricultural field soil:

For the isolation of soil fungi, soil dilution plate method was employed (Timonin, 1940). 10 gram of the collected soil sample was taken in a 250 ml of conical flask containing 100 ml of sterile water. The stock solution was thoroughly hand shaken or stirred for about 10 to 15 minute. The dilution was treated as 1: 100. Subsequently dilution i.e. 1: 10000 are prepared for the isolation of fungi. The dilution was done in the following way-

i.e. 1: 100 soil dilution ⟶ 1 ml dilution + 9ml Distilled water ⟶ 1ml dilution + 9ml of distilled water.

When the required dilution was prepared, 1ml of the solution were inoculated in the respective petridishes and then the rose Bengal agar media was poured in the petridishes (10 ml each approx) and shaken gently for few seconds and the media was allowed to solidify. After that the plates were incubated for 5- 7 days at 28±2° C. After the incubation period the growth of the fungus was observed and identified with the help of available literature (Burnett and Hunter, 1972; Raper and Fennel, 1973; Gilman, 1956; Nagamani *et al*., 2002). The total population of the fungi from the agricultural field soil was calculated by counting the number of colonies grown in the petridishes by the following formula:

$$\text{Total population} = \frac{\text{Total no. of colonies X Inoculum X Dilution factor}}{\text{Dry wt. of the Soil (gm)}}$$

Antagonism studies:

To investigate whether the antagonism existed between the test fungi and the pathogen (*Sclerotium rolfsii*), a 2mm disc of the antagonistic fungi was placed on the petridishes containing sterile PDA medium at 2cm apart from the pathogen. Three replicates were prepared for each fungus. Respective controls were also made. All the plates were separately incubated at 25±2° C for 7 days and the growth of the pathogen against the test fungi was measured in 2 days interval and at the same time the antagonistic colony interaction was also examined. The kind and degree of antagonism was determined according to the classification of Skidmore and Dickinsons (1976) as follows:

Colony interaction	Type of antagonism
Mutual intermingling growth	A
Overgrowth by antagonists	Bi
Intermingling growth in which the test fungus under observation has ceased growth and is overgrown by another colony	Bii
Light inhibition	C
Not detected	D

Test of Antagonistic potential:

Dual culture method (Wood, 1951) was followed for examining the antagonistic potential. 2mm discs of the pathogen and test fungus in triplicate on PDA from the 7 days old culture. The plates were then incubated

for 7 days at 25±1°C. Control plates were kept simultaneously. The colony grew on both sides i.e. towards and opposing each other from the loci was measured. The parameters used for the assessment of colony interaction were degree of inhibition or intermingled zone between both the colonies. The percentage inhibition of radial growth was calculated by using the equation given by Fokkema (1973).

Percentage of Inhibition, I= (C- T) × 100/ C

Where, C= Growth of pathogen in control (mm), T= Growth of pathogen in treatment (mm).

III. Results

Total population of the fungi:

The total population of fungi in the agricultural field soil was calculated by counting the number of colonies grown in the plates. The experiment was maintained in three replicates. The result **(Table 1)** shows that the soil of agricultural fields abounds luxuriant fungal population.

The result shows the dominant population of fungi isolated from the tea soil i.e. *Penicillium sp, Mucor sp, Aspergillus flavus, Aspergillus niger, Curvularia sp, Trichoderma harzianum, Trichoderma viride*, and *Fusarium sp* **(Table 2).**

Biological control: Six fungal species viz. *Aspergillus niger, Trichoderna harzianum, Trihoderma viride, Penicillium sp, Curvularia sp, and Fusarium sp* were tested to find out their antagonistic potential and the type of colony interaction against the test pathogen *Sclerotium rolfsii*. From the *in vitro* study it was observed that among the test fungi *Trichoderma harzianum* and *Trichoderma viride* are the most effective biocontrol agents. *Trichoderma harzianum* showed B_i type of interaction **(Table 3)** under which the antagonist inhibited the growth of the pathogen by overgrowing the mycelium of *Sclerotium rolfsii*. It shows that *Trichoderma harzianum* have the potential antagonistic effect against the *Sclerotium rolfsii* whose percentage of inhibition was, recorded to be 77.39% **(Table 4)**. The present findings of our study confirms with the observations made by Prabhu, Hirameth and Patil (1997). In case of *Trichoderma viride* the percentage of inhibition was found to be 76.54 %, followed by *Aspergillus niger* 30.48%, *Penicillium sp* 29.05%, *Fusarium sp* 13.57% and *Curvularia sp* 3.02% **(Table 4).** *Trichoderma viride* produced a clear inhibition zone showing the B_{ii} type of colony interaction **(Table 3)** in which the test fungus under observation has ceased to grow and it was overgrown by the antagonist *Trichoderma viride*. Although a clear inhibition zone was distinctly visible in some replicates, a slight overgrowth by the *Trichoderma viride* was also observed at the periphery of the colony.

Elad *et al*. (1983) reported that *T. harzianum* release B-1-3 Gluconase and Chitinase on the hyphal wall of *Sclerotium rolfsii* resulting in disintegration of the host mycelium which assists the penetration, growth, absorption, lysis and bursting of the host hyphae by the mycoparasite.

Aspergillus niger showed Type- C colony interaction and produced a slight inhibition zone against the *Sclerotium rolfsii* during the 4 days of incubation and later the colonies were overgrown by the pathogen. *Penicillium sp.* and *Fusarium sp*, were found to produce Type- A colony interaction under which mutual intermingling of growth was observed but it did not show any inhibition, ultimately leading to overgrowing by the pathogen. During 14 days of incubation, maximum reduction in the number of sclerotia was recorded in the presence of *T. harzianum* **(Plate 12)** followed by T. viride **(Plate 10)** as compared to Control and *Aspergillus sp* also reduced considerable number of Sclerotia production **(Plate 9)** but the growth of the colony was suppressed subsequently by the *Sclerotium rolfsii. Penicillium sp, Curvularia sp* and *Fusarium sp* could not compete with the pathogen and their growth was suppressed by the *Sclerotium rolfsii* and maximum production of Sclerotia was observed in the dual culture with them.

Table 1: Microbial population in agricultural field soil.

Replicates	No. of colonies	Mean	Total population
1	6	7.3	0.08×10^4
2	9		
3	7		

Table 2: Fungal colonies isolated and identified from the agricultural field soil.

Soil Sample	Sl. No.	Fungal species
Agricultural field soil	1	*Penicillium sp*
	2	*Mucor sp*
	3	*Aspergillus flavus*
	4	*Aspergillus niger*
	5	*Curvularia sp*
	6	*Trichoderma harzianum*
	7	*Trichoderma viride*
	8	*Fusarium sp*

Table 3: Types of colony interaction of the antagonist with the test fungus *Sclerotium rolfsii in vitro*:

Sl No.	Name of the Antagonist	Types of interaction
1	*Trichoderma harzianum*	Bi
2	*Penicillium sp.*	A
3	*Curvularia sp.*	D
4	*Aspergillus niger*	C
5	*Trichoderma viride*	Bii
6	*Fusarium sp.*	A

A: Mutual intermingling of growth, Bi: Overgrowth by the Antagonist, Bii: Intermingling growth in which the test fungus under observation has ceased to grow and is overgrown by another colony, C: Light inhibition, D: Not detected (Types of colony interaction as per Skidmore and Dickinson, 1976)

Table 4: Percentage inhibition of the radial growth of the pathogen by the antagonists *in vitro*:

Sl No.	**Name of the antagonist**	**Radial growth of the pathogen in (control plate)mm**	**Radial growth of the pathogen in dual inoculation (mm)**	**Percentage of inhibition**
1	*Trichoderma harzianum*	66.33(±0.27)	15.00(±0.77)	77.39%
2	*Penicillium sp.*	66.33(±0.27)	47.06(±0.19)	29.05%
3	*Curvularia sp.*	66.33(±0.27)	64.33(±0.10)	3.02%
4	*Aspergillus niger*	66.33(±0.27)	46.11(±0.27)	30.48%
5	*Trichoderma viride*	66.33(±0.27)	15.56(±0.78)	76.54%
6	*Fusarium sp.*	66.33(±0.27)	57.33(±0.31)	13.57%
7	Control	66.33(±0.27)		0%
				CD at 5% = 3.32
				CD at 1% = 5.20

Mean value, ± = SD, F- Test = 1.167(0.377)

Table 5: Mean growth of the Antagonists and the Pathogen (Sclerotium rolfsii) (mm) in Dual culture in vitro.

Antagonist + Pathogen	Antagonist(mm)	Pathogen(mm)
Trichoderma harzianum + S. rolfsii	66.267	15.033
Penicillium sp. + S. rolfsii	41.233	47.033
Curvularia sp. + S. rolfsii	32.833	64.333
Aspergillus niger + S. rolfsii	34.733	46.133
Trichoderma viride + S. rolfsii	66.667	15.567
Fusarium sp. + S. rolfsii	14.033	57.333
Control (only pathogen)	66.333	

Mean value, ± = SD

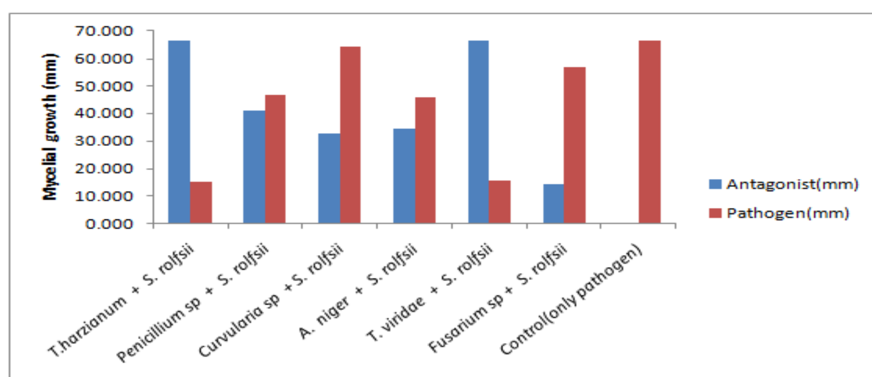

Fig 1: Bar diagram showing the comparison of mycelial growth of the antagonists against the pathogen in the dual culture in vitro.

IV. Discussion

Some soil fungi isolated from the agricultural field soil were found to grow fast in dual culture with the pathogen i.e., *Sclerotium rolfsii*. In the present study the slow growth rate of the pathogen suggested a more rapid utilization of nutrients by the antagonists when grown together. Nutrient depletion, space and production of toxic substances (antibiotic and antibiotic like substances) by the fungi are known to play a dominant role in antagonism and these factors are usually governed by the physico-chemical nature of the environment (Garret,

1963, Burgess and Griffin, 1967). The present in vitro study results showing the positive antagonistic effect of the soil fungi which have restricted the growth of the pathogen under *in vitro* condition (i.e, *Trichoderma sp.*)

The present observations show that the *Trichoderma harzianum* and *Trichoderma viride* are the most effective antagonist compared to other antagonists which were tested against the pathogenic fungus, i.e, (*Sclerotium rolfsii*). In case of *Aspergillus sp* and *Penicillum sp* very little inhibition of growth of the pathogen was observed. However, some of the antagonists tested were not found to be very effective against *Sclerotium rolfsii* under the in vitro observations but they may show better result under their natural field condition as their activities depends on the physico-chemical properties of the environment.

The inhibition shown by the antagonists may be due to the hyphal parasitism or the release of antibiotic/antibiotic like substances by the antagonists. Most of the soil fungi isolated from the agricultural field soil are known to survive saprophytically in nature. Release of inhibitory substances / metabolites produced by *Trichoderma harzianum* and *Trichoderma viride* into the host organism is known to result in direct inhibition of growth of the pathogen by disintegrating the hyphal wall resulting the penetration, absorption and lysis of the mycelium (Campbell, 1989; Wells *et al*., 1972). Due to adverse effect of chemical control of pest and diseases the attention of biological control has now increased by using some beneficial microorganisms. The application of biological controls using antagonistic micro-organisms has proved to be successful for controlling various plant diseases in many countries (Sivan, 1987).

Member of *Trichoderma* species are known to be active hyperparasites of several soil fungi and hence they are used as a biocontrol agents (Ekefan *et al*., 1990). Control of plant diseases by the use of antagonistic microorganisms can be an effective means (Cook, 1993). Various plant diseases have been successfully controlled through bacterial and fungal antagonists (Cook and Baker, 1980; Campbell, 1989).

Both antibiosis and parasitism play important role in the biocontrol of the plant diseases. There are two ways by which biocontrol agents can suppress the plant pathogen: (i) production of antibiotics or (ii) production of hydrolytic enzymes. Antagonistic microorganisms reduce growth, survival or infections caused by the pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. The exploitation of biocontrol agents for the management of plant diseases have achieved greater significance in the recent time due to its readily available nature, antimicrobial activity, easy biodegradability, non phytotoxicity, besides inducing resistance to the host.

To reduce the use of pesticides, biological control method has been considered as more natural and environmentally acceptable approach (Bagwan, 2010). *Trichoderma* was considered as a biocontrol agent for the phytopathogenic fungi, but the mechanism of this effect is not clearly understood. Proposed mechanisms of this biocontrol agent are thought to be antibiosis (Ghisalberti *et al*., 1990), mycoparasitism (Singh and Faull, 1990), competition or fungicidal action because of the capacity of *Trichoderma* for production of antibiotics or hydrolytic enzymes (Lorito *et al*., 1994). The *Trichoderma* species are capable of production of β-xylosidase, α-glycosidase, β- glycosidase, cellobiohydrolase, trypsin-, chymotrypsin- and chymoelastase-like proteases and N-acetyl- β-glucosaminidase, which are extracellular enzymes important for the biocontrol activity.

Antagonistic microorganisms, such as *Trichoderma*, reduce growth, survival or infections caused by the pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion (Ponnusamykonar *et al*., 2011). Several workers have investigated the use of biological control of plant diseases (Osando and Waudo, 1994; Tewari, 1995; Ogbebor and Adekunle, 2005). Osando and Waudo (1994) used various isolates of *Trichoderma* to control *Armillaria* root rot fungus of tea. They found that different isolates of *Trichoderma sp*. exhibited different level of antagonism against *Armillaria* root rot fungus.

The results obtained from the present work are similar with the findings of Deepak *et al*., (2008) where dry eye rot on apple caused by *Botrytis cinerea* were controlled under natural field conditions by the use of the antagonistic fungus *Trichoderma harzianum*. *Aspergillus spp.* which caused damage to *Colletotrichum gloeosporioides* by coagulating its cytoplasm also caused lysis and tip burst of the pathogen (Evueh *et al*., 2008).

From the in vitro findings, it can be suggested that the antagonists such as *Trichoderma harzianum, Trichoderma viride, Aspergillus niger* and *Penicillium sp* etc. can be used as a bio-control agent against *Sclerotium rolfsii* under field condition. It is also revealed that the microorganisms that naturally remain in the soil are having more or less similar potential antagonistic effect on the various crop disease caused by various pathogens. And some of them can be used as a potential bio- control agent under field condition to decrease the disease incidence and to increase crop productivity. Therefore, further work should be taken up to explore the possibility of the use of the antagonists under study in field condition for the biological control of the diseases caused by *Sclerotium rolfsii*.

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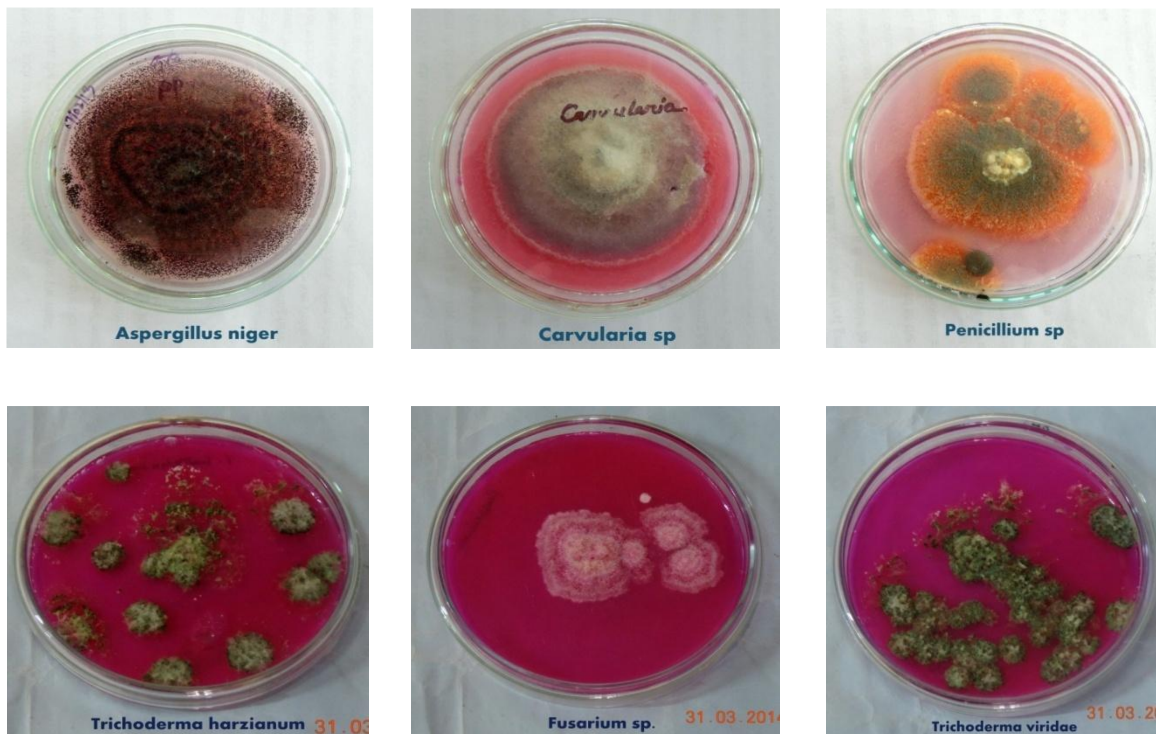

Plate 1: Pure culture of some of the isolated soil microorganisms (From Agricultural field soil).

Plate 2: Pure culture of the pathogen i.e. *Sclerotium rolfsii.*

Captions A= Antagonist, B= Pathogen and C= Control.

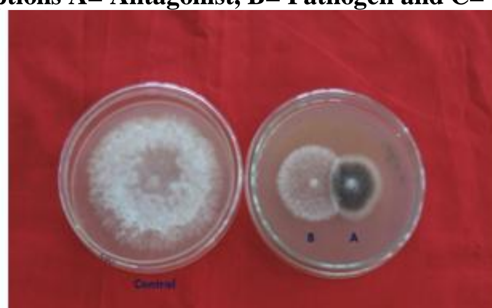

Plate 3: Antagonistic effect of A= *Aspergillus niger* against the pathogen B= *Sclerotium rolfsii in vitro*.

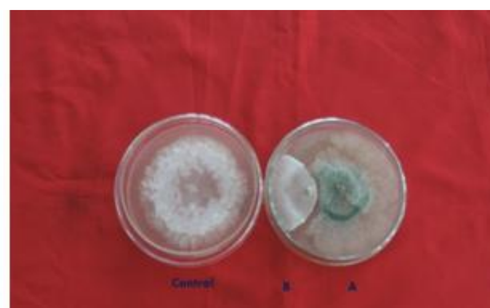

Plate 4: Antagonistic effect of A= *Trichoderma viridae* again the pathogen B= *Sclerotium rolfsii in vitro*.

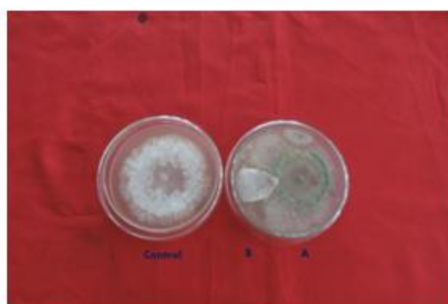

Plate 5: Showing the antagonistic effect of A= *Trichoderma harzianum* against the B= *Sclerotium rolfsii in vitro*.

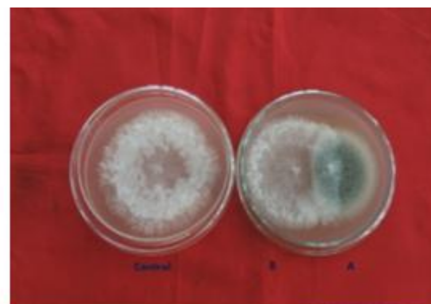

Plate 6: Showing the antagonistic effect of A= *Penicillium sp* against the pathogen B= *Sclerotium rolfsii in vitro*.

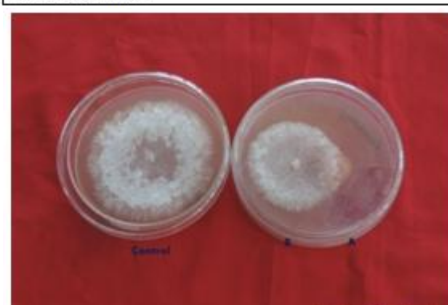

Plate 7: antagonistic effect of A= *Fusarium sp* against the pathogen B= *Sclerotium rolfsii in vitro*.

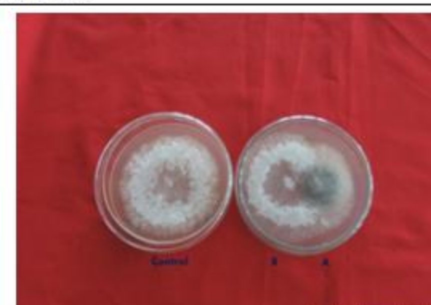

Plate 8: Antagonistic effect of A= *Curvularia sp* against the pathogen B= *Sclerotium rolfsii in vitro*.

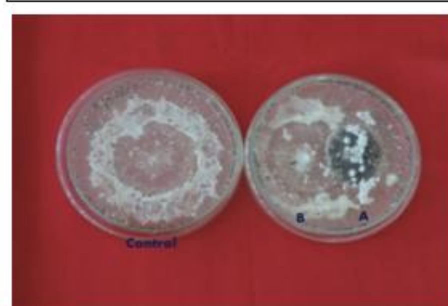

Plate 9: The antagonistic effect of A= *Aspergillus niger* in the sporulation of pathogen B= *Sclerotium rolfsii in vitro*.

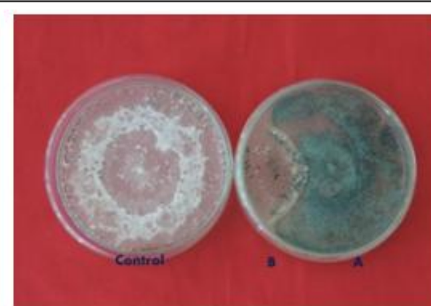

Plate 10: The antagonistic effect of A=*Trichoderma viride* on the sporulation of the pathogen B=*Sclerotium rolfsii in vitro*.

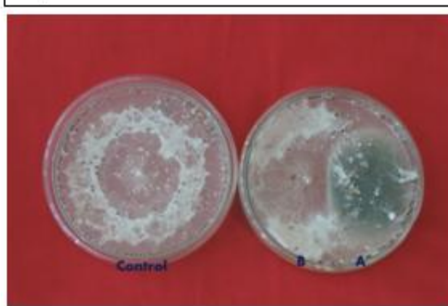

Plate 11: The antagonistic effect of A=*Penicillium sp* on the Sporulation of the pathogen B= *Sclerotium rolfsii in vitro*.

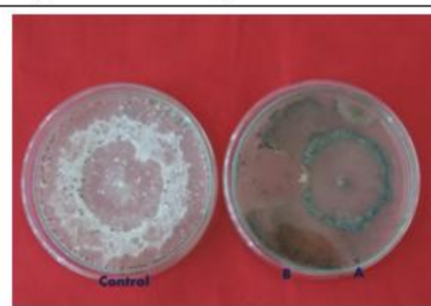

Plate 12: The antagonistic effect of A=*Trichoderma harzianum* on the growth of the pathogen B=*Sclerotium rolfsii in vitro*.

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