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BIOCONTROL EFFICACY OF GLIOCLADIUM VIRENS AND TRICHODERMA HARZIANUM AGAINST SCLEROTIUM ROLFSII SACC. A CAUSAL AGENT OF COLLAR ROT OF FIELD BEAN

S. Narasimha Rao[1*], S.L. Bhattiprolu[2] and B.M.V. Siva Prasad[3]

[1]Department of Plant Pathology, College of Horticulture, Dr. YSRHU, V.R. Gudem, [2]Regional Agricultural Research Station, ANGRAU, Lam, Guntur
[3]Ph. D. scholar, Department of Plant Pathology, Agricultural College, Bapatla, India

Email : varsha.snrao@gmail.com

ABSTRACT

Soilborne plant pathogens are of a major concern in agriculture which significantly reduces the crop yield. They can survive for many years in the absence of a host plant by forming persistent resting structures such as microsclerotia, sclerotia, chlamydospores and oospores. They are difficult to detect, predict, diagnose for successful disease management. Chemical control of soilborne plant pathogens imposes threats not only for soil fauna and flora but also potentially dangerous to humans as well as other animals. Biological methods in management of soilborne plant pathogens are more effective. *In vitro* study was conducted to assess the mechanisms of biocontrol by fungal antagonistic microorganisms against *Sclerotium rolfsii* in field bean. The evidence in supporting the mechanisms were metabolites produced by these organisms via a zone of inhibition with of interactions such as coiling of hyphae around the pathogen, penetration, and lysis of hyphae. Among the antagonists tested, *Gliocladium virens* and *Trichoderma harzianum* showed a strong antagonism by overgrew on mycelium and suppressed the growth of mycelium and production of sclerotia. Microscopic observation showed that vacuolation, coagulation of cytoplasm and aggregation of pathogen hyphae. Our results suggest that biological control of pathogens is an important factor in disease control in agriculture.

Keywords: Antagonists, biological control, collar rot, field bean, *Sclerotium rolfsii*.

INTRODUCTION

Field bean (*Dolichos lablab* L.) is one of the most ancient crops among cultivated plants, presently grown throughout the tropics, especially in South Asia and African countries (Rangaiah, 2016) and known different names worldwide *viz*., kidney bean, hyacinth bean, lab lab bean, bonavist field bean. Egyptian kidney bean, Indian butter bean, Australian pea, bonavist pea, dolique hyacinth bean, field bean, Indian bean or simply batao/batau, papaya bean, poor man's bean, seim bean, Tonga bean (White, 2015). In India it is known by different names in different parts; Telugu -Chikkudu, Tamil - Avarai, Marathi - Wal or Pavta, Hindi - Sem, Gujarati -Wal, Wal -papdi or Valor, Bengali - Shim, Malayalam - Mocca, Kanada – Chapparadaavare and Mai, Avaraa *etc*., (Shivashankar and Kulkarni, 1989). In Sanskrit राजशिम्बी (rājaśimbī), literally meaning royal legume (Aleksandar and Vesna, 2016) and cultivated for both pulse as well as vegetable crop for its tender pods, seeds and also for fodder. The productivity of field bean in farmer's field is rather low (1.2 t ha^{-1}) compared to its potential productivity (2.0 t ha^{-1}) under well managed production practices (Sylvester *et al*., 2016). Low productivity is due to both abiotic and biotic factors like pest and diseases causes enormous losses by reducing the yield, quantitatively and qualitatively. The crop is suffers from many diseases right from seed germination to crop maturity. Narayanan and Dabadghao (1972) listed the important disease of field bean in India. Among the fungal diseases, collar rot caused by *Sclerotium rolfsii* Sacc. (Saccardo, 1911) is an important necrotrophic, soilborne plant pathogen. *S. rolfsii* causes disease on thousands of plant species, including field, vegetable, fruit, and ornamental crops and survive in the soil by producing vegetative resting structure (selerotia) (Wydra, 1996). Collar rot on field bean was reported by Rao *et al*, (2002), Paula Junior *et al*, (2011), Chaurasia *et al*, (2014) and Tushar Ghevariya and Patel (2019).

Soil treatment with certain fungicides in the form of dusts, liquid drenches or granules are used to control damping-off, seedling blights, crown and root rots, and other diseases. Fungicides used for soil treatments include metalaxyl, diazoben, captan, chloroneb and fumigants like vapam and chloropicrin. Fumigants are toxic to sclerotia and mycelium in the soil. However, even after fumigation, some sclerotia survive, and treatments must be repeated annually. Thus biological control of soilborne plant pathogens by using different microorganisms helps to attain better plant growth and yield by the destruction of resting propagules. Biological control of plant diseases is the suppression of plant pathogen populations by living organisms (Heimpel and Mills, 2017).

There are two broad classes for plant disease control by antagonistic microorganisms. The first one is microbial antagonists occupy the same ecological niche as the target plant pathogen and interact directly with it. The mechanisms of interaction include hyperparasitism (Ghorbanpour *et al*., 2018), competition for space or food (Spadaro and Droby, 2016). Huge numbers of antibiotics are produced by actinomycetes (8700

different antibiotics), bacteria (2900) and fungi (4900) (Berdy, 2005), these antibiotics or other secondary metabolites that harm the target pathogen (Raaijmakers and Mazzola, 2012). The second class involves an indirect effect in which the control agent induces a resistance response in the plant that gives it protection against virulent plant pathogens. The 'inducer' for this form of control may use a particular strain of the plant pathogen that has low virulence, a different species of microorganism or a natural product, as well as the plant itself (Pieterse *et al*., 2014 and Conrath *et al*., 2015).

Several microorganisms have been exploited in biocontrol of soilborne plant pathogens (Berg, 2009). There are several fungal parasites on plant pathogens, including those that attack sclerotia (*i.e. Coniothyrium minitans*) while others attack living hyphae (*i.e. Pythium oligandrum*). Single fungal pathogen can be attacked by multiple hyperparasites, *Acremonium alternatum, Trichoderma viride, Acrodontium crateriforme, Ampelomyces quisqualis, Cladosporium oxysporum*, and *Gliocladium virens* (Heydari and Pessarakli, 2010).

MATERIALS AND METHODS

The causal agent responsible for collar rot on field bean was isolated from infected collar region of diseased plants by standard tissue isolation technique and identified as *S. rolfsii* based on morpho-taxonomic characters and maintained on potato dextrose agar (PDA). *In vitro* studies were conducted at Department of Plant Pathology, Agricultural College, Bapatla, on biocontrol efficacy of fugal antagonistic microorganisms on mycelial growth, sclerotial production and viability, size of sclerotia of *S. rolfsii*. The size of sclerotia was measured by placing them on graph paper and observed through hand lens. Fungal antagonistic microorganisms *viz., Trichoderma koningii*, *Trichoderma harzianum*, *Trichoderma viride*, *Gliocladium virens*, *Penicillium* sp, and *Aspergillus niger* were tested against *S. rolfsii.*

***In vitro* efficacy of fungal antagonists on mycelial growth:** The Petri plates containing 20 ml PDA medium were inoculated aseptically with five mm dia. culture blocks of *S. rolfsii* and fungal antagonist in opposite direction in such way that the distance between the two blocks of about 80 mm after solidification (Dennis and Webster, 1971).

Dual culture method was followed to study the biocontrol efficacy between the antagonists and *S. rolfsii* by growing them on cellophane membrane placed over solidified PDA in Petri plates. After both the fungi came in contact with each other, the contact zone was cut by using sharp blade and taken out along with cellophane. It was washed gently with water, mounted under lactophenol-cotton blue over a glass slide and observation was made under microscope (40X) (Dennis and Webster, 1971). Further small portion of intermingling area were randomly selected for microscopic observation to know the effect of antagonists. The sclerotia from 14 days old dual cultures were placed on PDA medium after washing with sodium hypochloride (1.0%) to test their viability.

These experiments were laid out in Completely Randomized Design (CRD) with three replications and test pathogen

alone at the centre served as control. All the Petri plates were incubated at 25±1°C temperature and observations on linear growth was recorded till the entire plate in control was covered by mycelium of *S. rolfsii*. Observation on radial growth was recorded by averaging the two diameters of colony at right angles to one another and per cent growth inhibition was calculated by using the formula given by Vincent (1947).

RESULTS AND DISCUSSION

Biocontrol of soilborne plant pathogens is considered as a potential management strategy in recent years, because chemical methods results in accumulation of harmful chemical residues, which may lead to serious ecological problems.

Efficacy of antagonists on mycelial growth and sclerotial production of *S. rolfsii*

The results presented in Table 1 indicated that, *T. harzianum*, *G. virens* and *T. viride* were equally effective and inhibited the radial growth 58.12%, 56.14% and 54.98% of *S. rolfsii*, respectively (Plate-1). These results are in line with the findings of Thiribhuvanamala *et al*, (1999) on the stem rot of tomato caused by *S. rolfsii*.

The antagonists secreted yellowish metabolite and formed an inhibition zone of varying size (2.12 to 3.59 mm). Maximum zone of inhibition was recorded with *T. harzianum* (3.59 mm) (Plate-2). The resulted zones of inhibition clearly visualize the effects of antagonists on *S.rolfsii* and are often used to explain the principles of biocontrol. This may be attributed to production of volatile or non volatile antibiotics by antagonistic microorganisms. The present findings were corroborate with the results of Ghorbanpour *et al*, (2018) who reported the *Trichoderma* and *Clonostachys* (former *Gliocladium*) produce 6-PAP, gliovirin, gliotoxin, viridin and many more compounds with antimicrobial activity.

T. harzianum (92.51%) and *T. viride* (90.74%) were inhibited the selerotia production of *S. rolfsii*. Present findings were in accordance with the findings of Virupaksha Prabhu *et al*, (1997). Sclerotia formed in the presence of antagonists were smaller (0.75 to 1.02 mm) than the control (1.89 mm). Smaller size of sclerotia indicated the lesser amount of reserve food material stored in them (Plate-4).

Interaction of antagonists with *S. rolfsii* in dual culture

Interaction between antagonists and *S. rolfsii* confirmed the involvement of different types of mechanisms in the present study via coiling, lysis, penetration, hyperparasitism (over growth), antibiosis and competition. In all the interaction except *A. niger* and *Pencillium* sp, test pathogen was antagonized by presence and activities of tested antagonistic microorganisms (Table 2).

a. Hyperparasitism- In this *S. rolfsii* is directly attacked by antagonists that kills or lysis the mycelium or its propagules (Plate 3 and 5). Reduction in growth of *S. rolfsii* was noticed when it was paired with antagonists. The difference in effectiveness of antagonists against *S. rolfsii* was observed after contact with each other. It was found that, *T. harzianum* and *G. virens* showed very strong antagonism, whereas *T. koningii* and *T. viride* showed moderate antagonism. While, *Penicillium* sp. and *A.*

niger showed no antagonism as it was not restricted the hyphal growth of *S. rolfsii*. Reisolation of *S. rolfsii* from the zone of interaction showed failure of *S. rolfsii* for production of mycelium. It may be due to lysis of pathogen hyphae by antagonists and also attributed the production of cell wall degrading enzymes combined with excretion of secondary metabolites in close contact with the *S. rolfsii* cell leading to openings in the cell wall and subsequent disorganization of the cytoplasm. Karlsson *et al*, (2017) and Nygren *et al*, (2018) reported the several biocontrol agents produce enzymes able to hydrolyze chitin, proteins, cellulose, and hemicelluloses contributing to direct suppression of plant pathogens.

b. **Antibiosis-** In the present study antagonists produced the volatile and or non volatile compounds that diffuse through PDA medium and suppressed the growth of *S. rolfsii*. In the present study interaction between *S. rolfsii* and antagonists indicated the antagonists produced certain compounds and passed through medium and inhibit/suppress the mycelial growth and production of inhibition zone (Plate-2). Further microscopic observations, four days after incubation in dual culture revealed the vacuolation and coagulation of cytoplasm and aggregation of hyphae of *S.rolfsii*. After seven days of interaction, lysis and rupturing of cell wall of *S. rolfsii* were observed (Plate 6). Volatile compounds from the antagonists have an important part of the inhibitory mechanism, especially under closed storage conditions. Paulitz *et al,* (2000) reported the production of volatile ammonia has been implicated as a possible mechanism to control soilborne pathogens. Kwee and Keng (1990) suggested the multiple mechanisms involving the mycoparasitism, antibiosis, lysis and hyphal interference in the interaction between *T. harzianum* and *S. rolfsii.*

c. **Competition-** The tested antagonists grew very fast, multiply and covered PDA medium on Petri plates for nutrients and space, which indicates antagonists have high competitive saprophytic ability. Antagonistic microorganisms protect the plant by rapid colonization, thus consumed the available substrates so that none is left for pathogens to grew, when they are applied to soil and there by restricted disease incidence. *S. rolfsii* is a necrotrophic plant pathogen kill host tissue well in advance and subsequently invade and utilize the available nutrients. Once necrosis occurred due to *S. rolfsii*, the antagonists with high competitive saprophytic ability colonize the necrotized tissues and able to occupy the niches and consumed nutrient sources rapidly as they were essential for pathogen infection. White hyphae of *Trichoderma* spp., at zone of inhibition gradually turned green and finally overgrew on *S. rolfsii* (Plate-3). These results were in conformity with the findings of Spadaro and Droby (2016) who reported wound protection of fruits from pathogen invasion by fast colonizing yeasts. *Trichoderma asperellum* producing iron-binding siderophores controls *Fusarium* wilt (Segarra *et al*., 2010). *Trichoderma* species are able to parasitize mycelium on infected debris and resting structure of plant pathogenic fungi in the soil, produce antibiotics, fungal cell-wall-degrading enzymes and

also they compete with soilborne pathogens for carbon, nitrogen and other factors, and they can also promote plant growth, possibly by the production of auxin like compounds (Vinale *et al*., 2008)

G. virens grew very rapidly, multiplied over it and growth of *S. rolfsii* was restricted completely. A dark green coloured growth with abundant sporulation was noticed over the pathogen. Coiling, lysis and attachment of hyphae of *G. virens* to the hyphae of *S.rolfsii* by hooks was observed (Plate-6).

A close observation on the surface of sclerotia revealed that, hyphae of antagonists penetrated through the rind of sclerotium and multiplied inside the sclerotium. Such parasitised sclerotia became dark, soft, empty and disintegrated under slight pressure. Antagonists penetrated into the sclerotia and replaced the internal contents by its mycelium and spores (Plate 5). These results were confirmed with the findings of Henis *et al*. (1983) and Suseelendra Desai and Schlosser (1999). The surface sterilized sclerotia in the presence of antagonists in dual culture fail to produce the mycelium when inoculated to Petri plates containing the PDA under aseptic conditions. This indicates the loss of viability resting spores of *S. rolfsii.* These results were supported by findings of Zheng *et al*. (2017) who confirmed that the viability of urediniospores of *Puccinia striiformis* f. sp. *tritici* from *A. alternata* treated pustules was only 25% whereas 80% of spores from untreated rust pustules were viable.

In the present study, the antagonistic microorganisms operate the different mechanisms in biocontrol of *S. rolfsii* through the hyperparasitism, production of antibiotics, through volatile and no volatile compounds, through competition for food, or through direct parasitizing the hyphae and sclerotia by lytic enzymes.

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Table 1. *In vitro* bio efficacy of antagonistic microorganisms on growth inhibition, number and size of sclerotia of *S. rolfsii*

Antagonistic Micro organisms	Per cent inhibition	Width of inhibition zone (mm)	No. of selerotia (14th day)	Per cent inhibition of sclerotia	Size of sclerotia (mm)
Trichoderma koningii	49.51* (44.71)**	2.17	13.50	88.45	0.75
Trichoderma harzianum	58.12 (49.66)	3.59	8.77	92.51	0.89
Trichoderma viride	54.98 (47.84)	2.47	10.83	90.74	0.92
Gliocladium virens	56.14 (48.51)	2.12	13.63	88.34	0.98
Penicillium sp.	22.14 (28.05)	No inhibition zone and over growth of *S. rolfsii*	85.77	26.63	1.72
Aspergillus niger	27.45 (31.58)	No inhibition zone and over growth of *S. rolfsii*	81.22	29.67	1.85
Control	0.00	-	116.90	0.00	1.89
CD at 1% level	2.33		5.06		
S.E(m) ±	0.75		1.65		
CV	3.10		6.30		

* Mean of three replications **Figures in parentheses are transformed (angular) values

Table 2. *In vitro* screening of antagonistic microorganisms against *S. rolfsii*

Antagonistic micro-organisms	Antagonism in dual culture	Mode of parasitism
Trichoderma koningii	++	Overgrew on test pathogen and covered the entire plate within 15 days of incubation
Trichoderma harzianum	+++	Overgrew on test pathogen and covered the entire plate within 12 days of incubation
Trichoderma viride	++	Overgrew on test pathogen and covered the entire plate within 15 days of incubation
Gliocladium virens	+++	Overgrew on test pathogen and covered the entire plate within 10 days of incubation
Penicillium sp.	-	Saprophytic activity, test pathogen overgrew on the antagonist
Aspergillus niger	-	Saprophytic activity, test pathogen overgrew on the antagonist
Pseudomonas fluorescens	+	Appear sticky growth of test pathogen and restrict the growth of test pathogen

- Overgrew of test pathogen on antagonists 10 days after incubation.

\+ Overgrew of test pathogen restricted by the antagonist.

++ Antagonists covered the entire plate within 15 days of incubation.

+++ Antagonists covered the entire plate within 10-12 days of incubation.

Plate-1 Inhibitory effect of antagonists on mycelial growth *S. rolfsii*

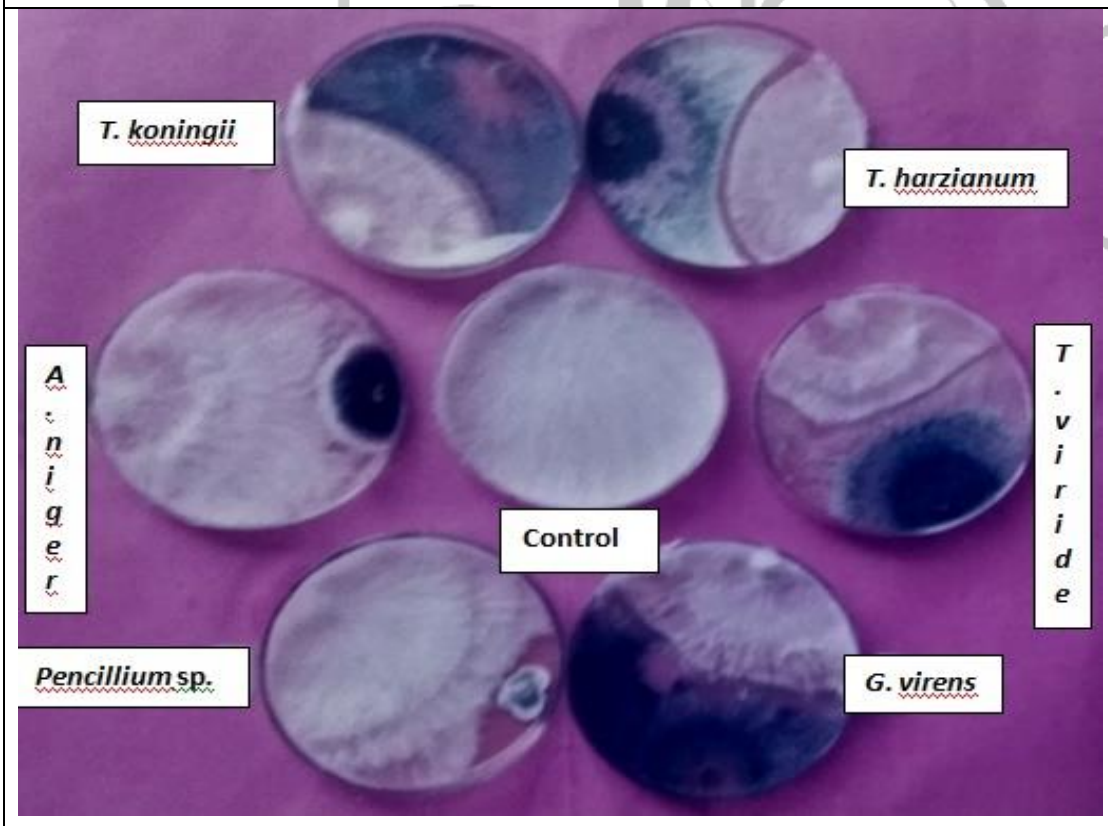

Plate-2 Production of zone of inhibition by antagonists against *S. rolfsii*

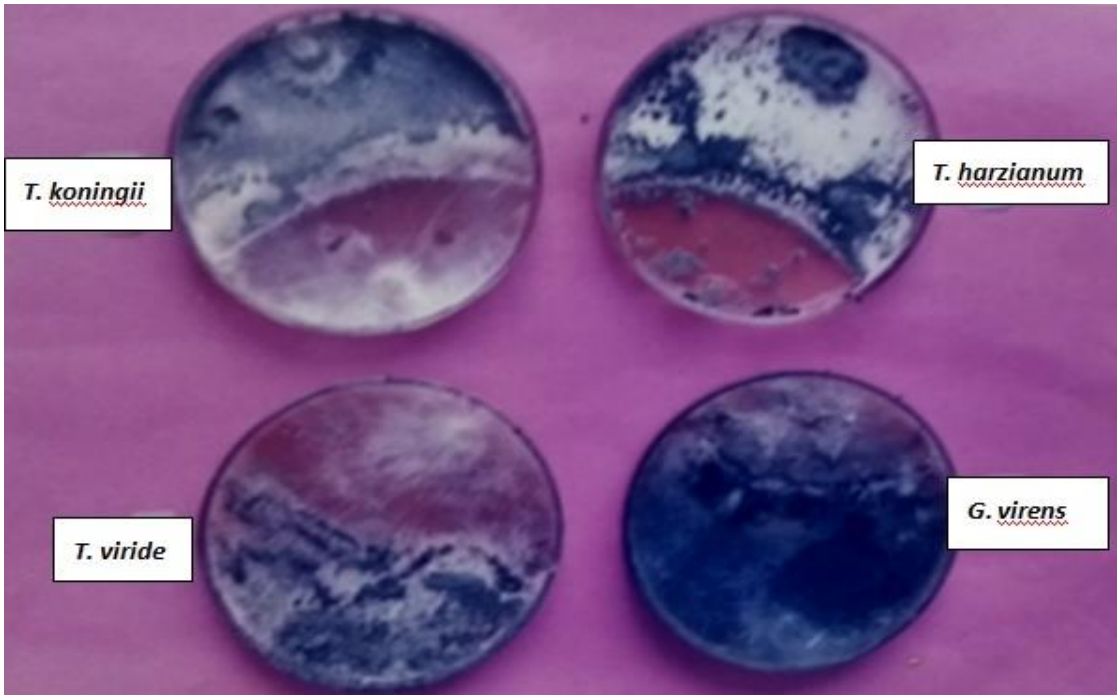

Plate-3 Hyperparasitism of fungal antagonists on *S. rolfsii*

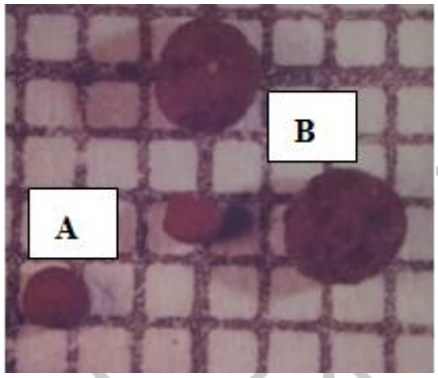

Plate-4. Size of sclerotia of *S. rolfsii* in the presence of antagonist (A) and control (B)

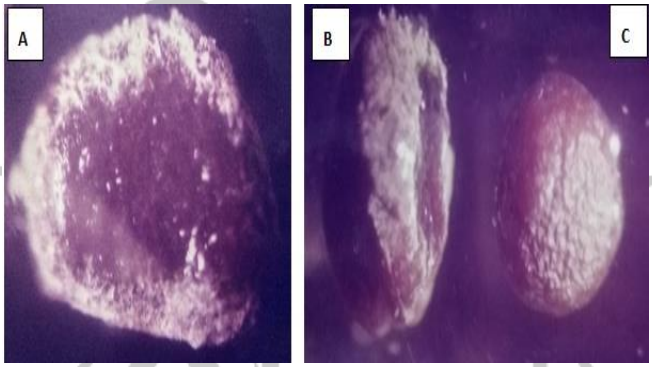

Plate-5 Micro photographs showing the parasitized (A) and disintegrated (B) and healthy sclerotium (C) of *S. rolfsii* by *T. harzianum* (40X)

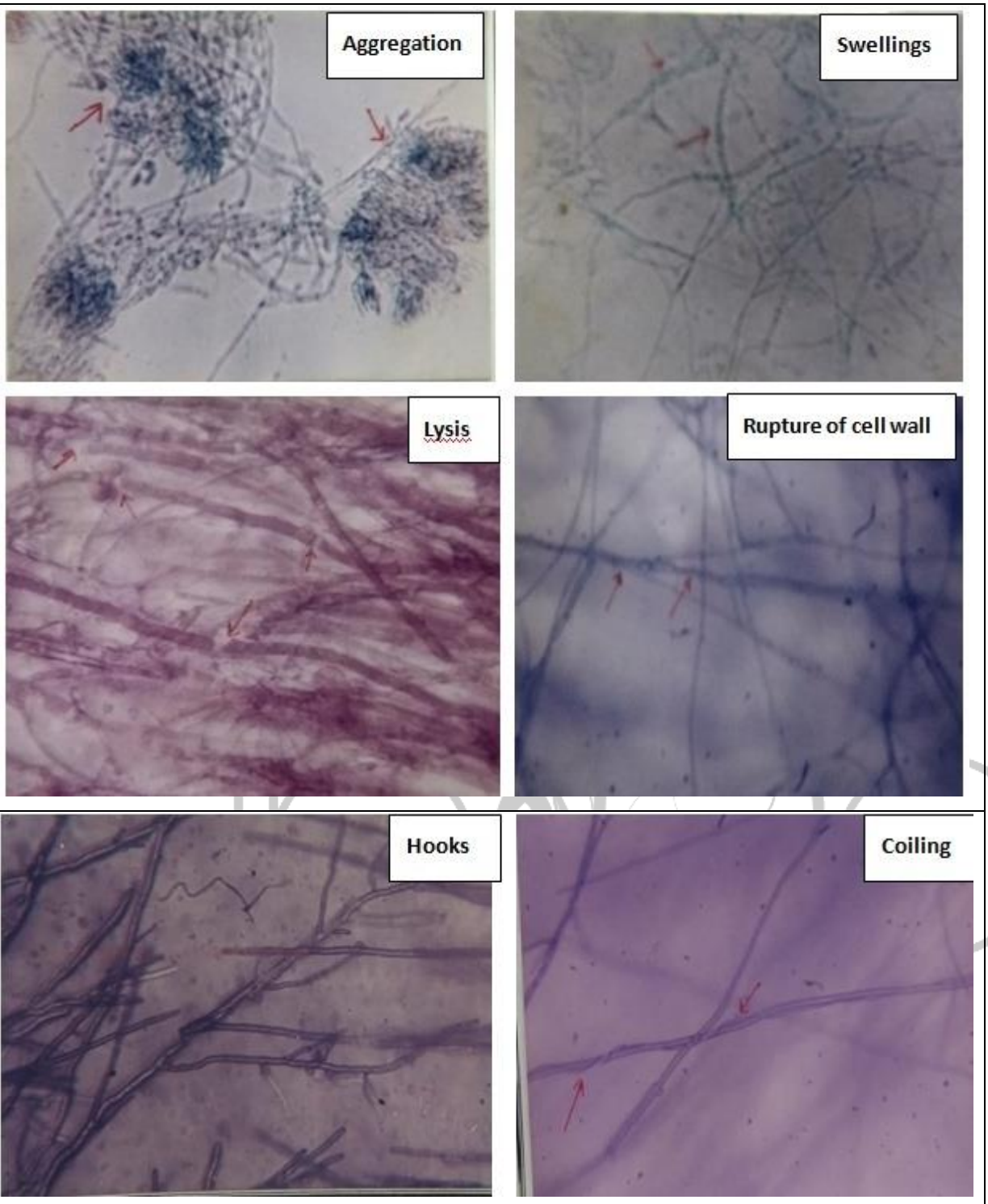

Plate-6 Micro photographs showing the effect of volatile and non volatile, cell wall degrading enzymes of *T. harzianum* on mycelium of *S. rolfsii* (40X)