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Impact of the Fungicide Rizolix T50% on the Antagonistic Activity of *Trichoderma harzianum* and *Trichoderma koningii*

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Abstract: ***Six Trichoderma spp. were isolated from the rhizosphere of maize and clover. All the isolates were able to tolerate Rizolex T 50%. Isolates of T. harzianum and T. koningii exhibited the best tolerance to this fungicide, reached to 350 and 400ppm respectively. The antagonistic effect of T. harzianum was high against pathogenic fungi, Fusarium oxysporum, Fusarium proliferatum and Acremonium strictum, reached to 77.3, 77.5 and 78% reduction respectively. While, T. koningii reduced the growth of these fungi to approximately 69, 73 and 77% respectively. The growth of all tested pathogenic fungi greatly decreased with increasing fungicide concentration in the growth medium comparing with the control. F. oxysporum and F. proliferatum tolerated Rizolex T50 % up to 450 - 550 ppm. whereas, Ac. strictum tolerated it only up to 250 ppm. The growth of T. harzianum and pathogenic fungi decreased with increasing Rizolex concentrations in the growth medium, moreover the antagonistic effect of T. harzianum against F. oxysporum, F. proliferatum and Ac. strictum at 200 ppm Rizolex T50% was approximately 86.6, 85 and 84% reduction respectively. Whereas, it was 74.3, 81.1 and 80% reduction respectively with T. koningii. Utilization of low dose of fungicide in combination with Trichoderma spp. increased the inhibition percentage of pathogenic fungi and may be beneficial in controlling these fungi.***

Keywords: Trichoderma species, Pathogenic fungi, Rizolex T50, Fungicides, Antagonistic activity.

1. Introduction

There is a worldwide trend to the use of ecologically safe methods of protecting crops from pathogens and pests. There is a growing concern, both in developed and developing countries including Egypt, about the use of hazardous fungicides for controlling fungal plant diseases. Now we know that the residues accumulation of fungicides in the soil and water interferes with numerous biological activities[1].

Biological control is becoming an urgently needed component in agriculture. Chemical pesticides have been the object of substantial criticism in recent years. Therefore, it is important to develop safer and environmentally feasible control alternatives, mainly by the use of existing living organisms in their natural habitat. These organisms are able to provide protection against a large range of plant pathogenic fungi without damage to the ecological system[2].

Fusarium proliferatum, a relatively described species occurs worldwide on a broad variety of plants including maize[3]**.** Two species *F. proliferatum* and *F. verticilloides* were considered as the more widespread on maize, sorghum and many other cereals. These two species were found associated with all plants growth stages [4],[5].

Rizolex T 50% is non systemic contact fungicide with protective and curative action. Acts by inhibition of phospholipids biosynthesis, leading to inhibition of germination of spores and growth of fungal mycelium. Control of *Fusarium* by chemicals is often uneconomical and has negative environmental impacts and development of fungicidal resistance variety [6]. The most important alternative method to chemical control of disease causing pathogens like *Fusarium*, *Pythium* and *Rhizoctonia* was achieved by applying biological control [7],[8].

The antagonistic ability of *Trichoderma asperellum*, *T. atroviride*, *T. koningii*, *T. harzianum* and *T. reesi* against *Fusarium oxysporum* f. sp. *phaseoli* was evaluated under lab. and green house conditions and found that *T. reesei and T. koningii* was the most effective isolates against the pathogen and for stimulation of plant growth[6]. *T. harzianum*, *T. asperellum*, *T. virens* against *F. oxysporum* of lentil was evaluated and found that all of them could effectively inhibit growth of the fungus in laboratory tests. Three isolates alone and in combination in green house revealed that disease severity was reduced from 20 to 44% while, dry weight increased from 23 to 52% when *T. harzianum* and *T. asperellum* were combined [9]. The effectiveness of two *Trichoderma* isolates *Trichoderma viride* and *Trichoderma Koningii* against *Fussarium oxysporum* causing root rot in beans was studied in vitro and under greenhouse condition. They founded that nearly 100% inhibition of *Fusarium oxysporum* growth by antagonistic *T. viride* and very poor growth of *Fusarium* with a reduction of 91% was observed in the presence of antagonist *T. koningii* [10].

Trichoderma asperellum, *T. harzianum*, *T. harzianum* and *T. hamatum* and a low dose of seed fungicides Rizolex-T50% and Celest-max in combination were used to detect compatible fungicide/antagonist combinations for integrated disease control. Results showed that the combinations did not increase the efficacy against stem cancer of potato. however, effectiveness of fungicide/antagonist combinations on black scurf was higher with limited extent[11]. This work aims to evaluate the antagonistic activity of Trichoderma spp. against pathogenic fungi in absence and in the presence of fungicides

2. Materials and methods

2.1 Isolation of Trichoderma spp. from Egyptian soil.

Trichoderma spp. was isolated by dilution technique method from soil obtained from rhizosphere of healthy and diseased plants (maize and clover). A serial dilution was made for each sample up to 1/10000. 0.5 ml from each dilution was inoculated on to potato dextrose agar plates containing rose Bengal and streptomycin [12]. The plates were incubated at 30°C for 4 days. All plates were examined daily and different *Trichoderma* spp. were isolated and purified on a new PDA plates.

Screening the sensitivity of isolated *Trichoderma spp.* toward Rizolex T 50%.

Different concentrations of fungicide was added separately to 100 ml of sterilized PDA medium immediately after cooling before solidification under aseptic condition and mixed thoroughly to give the required concentrations. The control was maintained without fungicide. Prepared medium was aseptically poured in sterilized Petri dishes, Three replicates were used for each treatment. Equal disks (8 mm diameter) from *Trichoderma* 7 days old culture were placed onto the middle of the agar plate. Inoculated plates were incubated at 30°C and mycelial growth was measured daily. The best *Trichoderma* spp. which tolerated high concentrations of fungicides was selected for further studies.

Identification of selected *Trichoderma spp.:*

Trichoderma spp. was identified by the regional center of mycology and biotechnology, Al-Azhar University, Egypt. They were identified as *Trichoderma harzianum* and *Trichoderma koningii* (Fig.1), where pathogenic fungi *Fusarium proliferatum*, *Fusarium oxysporum* and *Acremonium strictum* were obtained as a gift from plant pathology research institute, Agriculture research center, Giza, Egypt.

Antagonistic effect of selected Trichoderma spp. against pathogenic fungi:

T. harzianum and *T. koningii* were grown against the pathogens *Fusarium oxysporum*, *Fusarium proliferatum* and *Acremonium strictum* to test their antagonistic potential on Potato Dextrose Agar (PDA). Eight mm disk of seven-day-old cultures of Pathogenic fungi and *Trichoderma* were placed against each other on plates. In case of control instead of *Trichoderma* disk, PDA disk was used. The plates were incubated at 30ºC for 7 days. Radial growth of the pathogens and *Trichoderma* was measured and data were obtained. Data of laboratory tests were calculated by the following formula:

% inhibition = {(Diameter of fungal growth in control - Diameter of fungal growth in treatment)/ Diameter of fungal growth in control} × 100.

Effect of different concentrations of Rizolex T50% on radial growth of selected *Trichoderma* spp. and pathogenic fungi:

PDA medium was amended with different concentrations of Rizolex T 50% (0, 50, 100, 150 up to 550 ppm), 10 ml of medium at 45^0C was poured to sterilized Petri dishes under aseptic condition and allowed to solidify. After solidification, 8 mm circular disc of 10-day-old pathogenic fungi and selected *Trichoderma* spp. was transferred aseptically to the center of agar plates. The Petri plates were incubated at 30°C for 8 days, radial growth was measured daily.

3. Results

Six *Trichoderma* spp. were isolated from rhizosphere of maize and clover. Growth of all isolated *Trichoderma* spp. was highly decreased with increasing fungicide concentrations in the growth medium comparing with the control. All *Trichoderma* isolates were able to tolerate and grow on the agar medium containing 100ppm of Rizolex T 50%. T5 and T6 were the isolates which are able to tolerate high concentrations of Rizolex T 50% reached to 350 and 400ppm respectively (Table 1).

Antagonistic activity of selected Trichoderma species against pathogenic fungi.

In vitro antagonistic property studies were carried out to evaluate the efficiency of *Trichoderma harzianum* and *T. koningii* on the growth of the phytopathogenic fungi *Fusarium oxysporum, F. proliferatum and Acremonium strictum* by direct competitive interaction. The growth of pathogenic fungi in absence of *Trichoderma spp.* reached to approximately 75 mm colony diameter for *F. oxysporum* and *F. proliferatum* and 50mm for *Ac. strictum* whereas; in the presence of *T. harzianum* it sharply decreased to 17, 20 and 11 mm respectively. Therefore its antagonistic effect was very high and reached to 77.3, 77.5 and 78% reduction in the growth of the pathogenic fungi respectively. On the other hand, *T. koningii* reduced the growth of pathogenic fungi *F. oxysporum*, *F. proliferatum* and *Ac. strictum* to approximately 69, 73 and 77% respectively (Table 2 and Fig. 2).

Effect of different concentrations of Rizolex T 50 % on radial growth of selected *Trichoderma* isolates

The growth of both *T. harzianum* and *T. koningii* was markedly decreased with increasing fungicide concentrations in the growth medium at different incubation periods. *T. harzianum* was able to grow in the presence of Rizolex T50% up to 400 ppm with percentage inhibition of 83.3% while *T. koningii* cannot grow at this concentration but grown up to 350 ppm with percentage inhibition of 87.7% after six days. 50% inhibition of *T. harzianum* was obtained after six days of incubation period at 100ppm of Rizolex T 50 % while it was obtained at 50 ppm for *T. koningii*. At concentration of 350ppm of Rizolex T 50 %, the growth of *T. harzianum* was greatly reduced to approximately 78.8% whereas, it reduced to approximately 87.7% for *T. koningii*, this indicated that *T. koningii* was

more sensitive to Rizolex T 50 % than *T. harzianum* (Table 3).

Radial growth of Pathogenic fungi at different concentrations of Rizolex T 50 %:

Fusarium proliferatum, *Fusarium oxysporum* and *Acremonium strictum* exhibited a different ability to grow on different concentrations of Rizolex T 50 % in growth medium at different incubation periods. The growth of all tested pathogenic fungi greatly decreased with increasing fungicide concentration in the growth medium comparing with the control. After eight days, *F. oxysporum* and *F. proliferatum* were able to grow at 450, 550 ppm respectively of Rizolex T 50 % with percentage inhibition of 84.4% and 78.8% and they cannot able to grow above these concentrations. In contrast, *Ac. strictum* was the most sensitive one to Rizolex T 50 %, as it was not able to grow above 250 ppm of Rizolex T 50 % with percentage inhibition 80% at this concentration after eight days. 50% inhibition of *F. proliferatum* was approximately obtained at 250ppm of Rizolex T 50 %, while it was obtained at 150 ppm for *F. oxysporum* and less than 50 ppm for *Ac. strictum*, (Table 4).

Antagonistic activity of *T. harzianum and* T. koningii toward the pathogenic fungi in the presence of different concentrations of Rizolex T 50%:

The growth of pathogenic fungi in absence of *T. harzianum* in the control medium without Rizolex T50% reached to approximately 75mm colony diameter for *F. oxysporum* and *F. proliferatum* and 50mm for *Ac. strictum* whereas, in the presence of *T. harzianum* it sharply decreased to 17, 20 and 11 mm respectively. Therefore its antagonistic effect was very high and reached to 77.3, 77.5 and 78% reduction in the growth of the pathogenic fungi respectively (Table 5).

The presence of Rizolex T 50% in the growth medium highly decreased the growth of *T. harzianum* and pathogenic fungi. The antagonistic effect of *T. harzianum* against pathogenic fungi slightly increased in the presence of the fungicide Rizolex T50%. At 200 ppm Rizolex T in combination with *T. harzianum* reduced the growth of *F. oxysporum* and *F. proliferatum* and *Ac. strictum* to approximately 86.6, 85 and 100% respectively. While, with *T. koningii* in combination with Rizolex T at 300 ppm, the growth of these pathogenic fungi reduced to approximately 80. 81.1 and 100% respectively. Utilization of low dose of fungicide in combination with *Trichoderma spp*. increased the inhibition percentage of pathogenic fungi and may be beneficial in controlling these fungi.

4. Discussion

In recent years, research on biological control has gained momentum for controlling serious soil born plant pathogens like *Fusarium, Rizoctonia ,Macrophomina, Sclerotium, Pythium and Phytophthora spp*, employing *Trichoderma* and *Gliocladium* species and varied success has been achieved**.** Several *Trichoderma spp*. could be effectively used in biocontrol of soil borne plant pathogens, *Trichoderma* spp. are active as hyperparasites [13].

In this work, the antagonistic activity of isolated *Trichoderma harzianum* was high against pathogenic fungi *F. oxysporum*, *F. proliferatum* and *Ac. strictum* and reached to 77.3, 77.5 and 78% reduction respectively. While, *T. koningii* reduced the growth of these fungi to approximately 69, 73 and 77% respectively. Several reports have indicated that biocontrol efficiency of *Trichoderma spp.* against *Fusarium* wilts may differ in different regions of the world i.e., a highly antagonistic species against a particular pathogen in a given region may react poorly against the same pathogen in another region[14],[15]. In a study carried out by [16], the efficacy of *T. koningii* for the suppression of *F. oxysporum* reached to about 91% in vitro while, in this study, *T. koningii* was able to reduce it to 69.3%.

T. harzianum was able to grow in the presence of Rizolex T50% up to 400 ppm with percentage inhibition of 83.3% while, *T. koningii* cannot grow at this concentration but grown up to 350 ppm with percentage inhibition of 87.7%. Similar result was also observed by [17]**,** they observed that mycelial growth of *Trichoderma* spp. was most sensitive to Benlate, Ridomil gold, Tecto-60 and Topsin-M at both the concentrations of 100 and 200ppm. Similar results concerning the inhibitor effect of Rizolex-T 50% and Topsin-M at different concentrations against soil borne fungi, *R. solani* and *F. oxysporum* were also observed by [18]**.**

In our results the combination of *Trichoderma spp*. with low concentration of Rizolex T lead to increase inhibition of pathogenic fungi (*F. oxysporum, F. proliferatum* and *A. strictum*) in compare with *Trichoderma* or low concentration of Rizolex T separately. This result agrees with [19], they reported that the efficacy of the *Trichoderma* treatments has been both more variable and less effective than chemical fungicide treatments. Combining bio-control agents with selective fungicides that are not inhibitory to the antagonist will help it to become established in the soil and achieve better disease control and also with[20], who demonstrated an improved disease control of gladiolus corm rot and wilt caused by *F. oxysporum* by combining an isolate of *T. virens* and the fungicide carboxin. Analogous results were obtained by [21]**,** with mutants of benomyl-tolerant strains of *T. pseudokoningii*, which were superior to the wild type in bio-control potential on S. rolfsii.

Apparently, biocontrol with *Trichoderma* has to be implemented with other disease control measures to reach a satisfactory level of disease control. A correlation between fungicide resistance and antagonistic activity is suggested by [22]**,** affirming that the up regulated expression of ABC transporter genes of *T. atroviride* during the three-way interaction with various plants and fungal pathogens, possibly supports both antagonistic activity and root colonization.

In concolusion: Isolation and selection of fungicides tolerant strains of *Trichoderma* species may help in better controlling of pathogenic fungi. Application of low doses of rizolex T50% in combination with biologically control *Trichoderma* species highly increased the inhibition ratio of pathogenic fungi and therefore, better control of plant diseases.

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Table 1: Radial growth (mm) of ***Trichoderma* isolates** grown on PDA medium amended with different concentrations of **Rizolex T 50 %** fungicides after six days. Data are the mean value of 3 replicates ± standard error of mean.

Rizolex T 50 % (PPM)	T1	T2	T3	T4	T5	T6
0	87±0.10	90±0.00	90±0.00	90±0.00	90±0.00	90±0.00
50	20±0.08	17±0.08	23±0.04	28±0.10	41±0.04	60±0.04
100	13±0.08	11±0.04	16±0.10	22±0.11	32±0.08	44±0.08
150	0.0	0.0	11±0.08	14±0.10	28±0.08	35±0.08
200	0.0	0.0	0.0	9±0.04	23±0.10	32±0.12
250	0.0	0.0	0.0	0.0	20±0.04	29±0.04
300	0.00	0.0	0.0	0.0	18±0.04	23±0.04
350	0.00	0.0	0.0	0.0	11±0.08	19±0.08
400	0.00	0.0	0.0	0.0	0.0	15±0.04

Table 2: Antagonistic effect of *T. harzianum* and *T. koningii* against pathogenic fungi, Data are expressed as colony diameter (mm) after 6 days of incubation, mean of 3 replicates ± standard error of mean.

	Trichoderma against F. oxysporum			*Trichoderma against F. proliferatum*			*Trichoderma against AC. strictum*		
	Trichoderma	*F. oxysporum*	% reduction	*Trichoderma*	*F. proliferatum*	% reduction	*Trichoderma*	*Ac. strictum*	% reduction
Control	--	75±0.10	--	--	75±0.10	--	--	50±0.08	--
T. harzianum	39±0.12	17±0.12	77.3	38±0.06	20±0.12	73.3	40±0.06	11±0.06	78
T. koningii	30±0.10	23±0.07	69.3	31±0.12	20±0.12	73.3	35±0.03	17±0.10	77.3

Table 3: Effect of different concentrations of Rizolex T 50 % on radial growth of *T. harzianum* and *T. koningii*. Data are the mean value of 3 replicates ± standard error of means.

Fungicide concentration	*T. harzianum*				*T. koningii*			
	2 day	4 day	6 day	Inhibition%	2 day	4 day	6 day	Inhibition%
0 ppm	58±0.07	83±0.07	90±0.00	00	55±0.08	80±0.04	90±0.00	00
50 ppm	25±0.06	43±0.03	60±0.04	33.3	14±0.08	28±0.08	41±0.04	54.4
100 ppm	18±0.12	30±0.06	44±0.08	51.1	12±0.08	21±0.08	32±0.08	64.4
150 ppm	13±0.10	24±0.07	35±0.08	61.1	11±0.00	19±0.08	28±0.08	68.8
200 ppm	12±0.10	23±0.10	32±0.12	64.4	8±0.00	16±0.08	23±0.10	74.4
250 ppm	11±0.10	20±0.09	29±0.04	67.7	8±0.00	12±0.00	20±0.04	77.7
300 ppm	10±0.05	16±0.09	23±0.04	74.4	8±0.00	12±0.08	18±0.04	80
350 ppm	8±0.03	13±0.09	19±0.08	78.8	8±0.00	9±0.03	11±0.08	87.7
400 ppm	8±0.00	10±0.09	15±0.04	83.3	8±0.00	8±0.00	8±0.00	-

Table 4: Effect of different concentrations of Rizolex T 50% on radial growth of *Fusarium proliferatum*, *Fusarium oxysporum* and *Acremonium strictum.* Data are the mean value of 3 replicates ± standard error of means.

Fungicide conc.	*F. proliferatum*				*F. oxysporum*				*Ac. strictum*			
	4 day	6 day	8 day	I%*	4 day	6 day	8 day	I%	4 day	6 day	8 day	**I%**
0ppm	49±0.0	68±0.08	90±0.0	00	57±0.0	79±0.09	90±0.0	00	31±0.07	49±0.0	63±0.0	**00**
50ppm	34±0.0	49±0.08	65±0.0	27.7	23±0.0	39±0.03	55±0.0	38.8	15±0.07	23±0.0	31±0.0	**65.5**
100ppm	28±0.0	42±0.04	57±0.0	36.6	20±0.0	32±0.06	48±0.0	46.6	13±0.03	17±0.0	24±0.0	**73.3**
150ppm	24±0.0	37±0.04	50±0.0	44.4	17±0.1	27±0.07	40±0.0	55.5	11±0.07	15±0.0	21±0.0	**76.6**
200ppm	21±0.1	33±0.08	47±0.0	47.7	14±0.0	25±0.07	36±0.0	60.0	9±0.03	12±0.0	18±0.0	**80**
250ppm	19±0.0	30±0.08	44±0.0	51.1	12±0.0	20±0.03	32±0.0	64.4	8±0.03	9±0.07	11±0.0	**87.7**
300ppm	16±0.1	27±0.10	40±0.1	55.5	11±0.0	19±0.03	29±0.0	67.7	0.00	0.00	0.00	
350ppm	14±0.0	25±0.04	36±0.0	60	11±0.0	16±0.03	26±0.0	71.1				
400ppm	12±0.1	21±0.12	34±0.1	62.2	10±0.0	14±0.07	20±0.0	77.7				
450ppm	11±0.0	18±0.04	29±0.0	67.7	10±0.0	11±0.03	14±0.0	84.4				
500ppm	10±0.0	14±0.08	23±0.0	74.4	0.00	0.00	0.00					
550ppm	8±0.04	12±0.04	19±0.0	78.8								

* **I% expressed** inhibition percentage

Table 5: Antagonistic effect of *Trichoderma harzianum* and *T. koningii* against pathogenic fungi in the absence and presence of fungicide. Data are expressed as colony diameter (mm) after 6 days of incubation, mean of 3 replicates ± standard error of mean.

Trichoderma strains	Rizolex (ppm)	*Trichoderma*	*F. oxysporum*	% reduction	*Trichoderma*	*F. proliferatum*	% reduction	*Trichoderma*	*Ac. strictum*	% reduction
T. harzianum	Cont.	-	75±0.10	-	-	75±0.10	-	-	50±0.08	-
	0	39±0.12	17±0.12	77.3	38±0.06	20±0.12	73.3	40±0.06	11±0.06	78
	100	29±0.12	15±0.15	80	32±0.06	20±0.12	75	30±0.06	10±0.07	80
	200	24±0.32	10±0.15	86.6	21±0.23	13±0.10	85	25±0.12	-	100
	300	20±0.12	11±0.07	85.3	20±0.18	12±0.12	86.3	22±0.07	-	-
	400	16±0.12	11±0.07	85.3	17±0.18	12±0.10	86.3	-	-	-
T. koningii	Cont.	-	75±0.10		-	75±0.10		-	50±0.08	
	0	30±0.10	23±0.07	69.3	31±0.12	20±0.12	73.3	35±0.03	17±0.10	77.3
	100	25±0.12	24±0.12	68	24±0.06	18±0.06	76	25±0.03	11±0.23	78
	200	20±0.09	19±0.12	74.3	20±0.15	14±0.12	81.1	21±0.12	10±0.25	80
	300	14±0.06	15±0.06	80	15±0.23	14±0.15	81.1	15±0.12	-	100

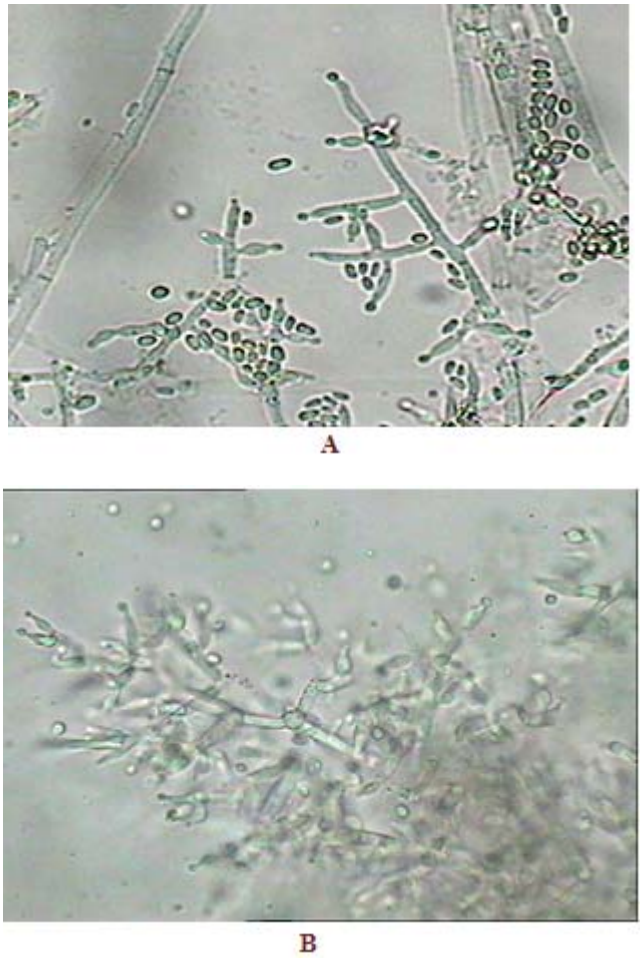
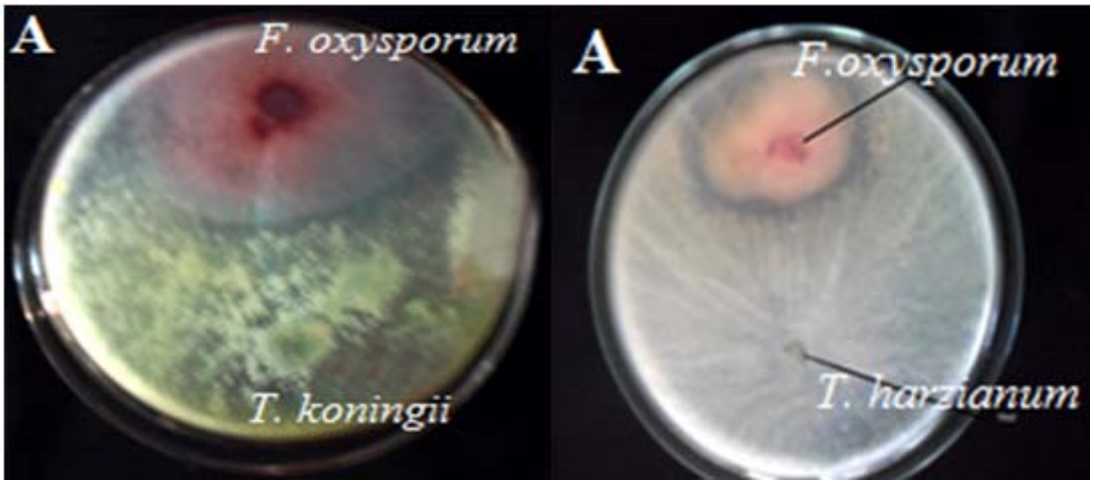


Figure 1: Photomicrograph shows the phialides and conidia of (A) *Trichoderma koningii* and (B) ***Trichoderma harzianum*** under microscope.



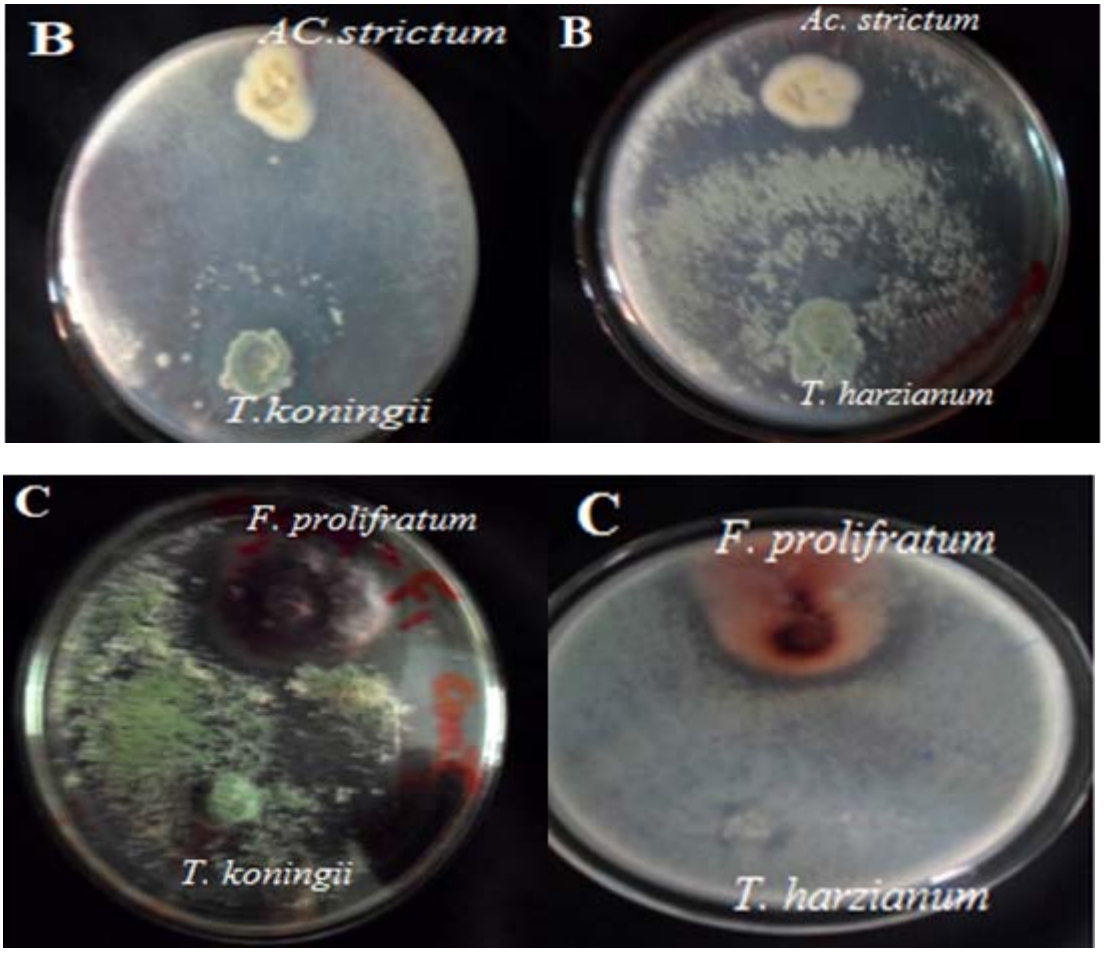


Figure 3: Photograph shows antagonistic activity of *T. koningii* and T. harzianum against pathogenic fungi (**A**) *F. oxysporum*, (**B)** *Ac. strictum and (***C)** *F. proliferatum.*