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IN VITRO ANTAGONISM OF *Trichoderma* ISOLATES AND EFFICACY OF CHEMICAL FUNGICIDES AGAINST MYCELIAL GROWTH OF *Pestalotiopsis theae*

Karun Adhikari*, Anupam Raj Khadka & Kailash Shrestha
Himalayan College of Agricultural Sciences and Technology, Nepal
* Corresponding author's email: karunadhikary45@gmail.com
Karun Adhikari https://orcid.org/0009-0003-1094-9607
Anupam Raj Khadka https://orcid.org/0009-0005-5521-8044
Kailash Shrestha https://orcid.org/0009-0000-3843-5438

ABSTRACT

Grey leaf blight, one of the most important fungal foliar diseases of tea is caused by Pestalotiopsis spp. An in vitro study was conducted at Agriculture Research Station, Pakhribas to evaluate bio-efficacy of two Trichoderma isolates namely Trichoderma viride and Trichoderma harzianum using dual culture technique. Similarly comparative efficacy of five fungicides viz. Copper Oxychloride 50% WP, Carbendazim 50% WP, Metalaxyl 8% WP + Mancozeb 64 % WP, Carboxin 37.5% WS + Thiram 37.5% WS and Hexaconazole 5 % EC at four different concentration of 50 ppm, 100 ppm, 200 ppm and 500 ppm was tested using poisoned food technique. The experiment was arranged in a completely randomized design with three replications for each treatment and a control. Trichoderma viride and Trichoderma harzianum exhibited 66.64% and 62.32% growth inhibition respectively against Pestalotiopsis theae. Growth inhibition by fungicides ranged from 35% to 100%. Hexaconazole exhibited complete mycelial growth inhibition (100%) regardless of concentration. This complete inhibition (100%) in colony growth was recorded at 200 ppm and 500 ppm of Carboxin + Thiram and then in Metalaxyl + Mancozeb at 500 ppm.While least (35.62%) inhibition on mycelial growth of P. theae was observed with Copper Oxychloride at 50 ppm. There was significant reduction in colony growth of P. theae with each treatment at all concentration in comparison to control. This study indicated significant results by fungicides even at lower concentration and by both Trichoderma spp. This study established Trichoderma spp as a successful alternative and hence recommends use of an integrated approach with both effective fungicides at low concentration and bio-control agents in further managing grey leaf blight of tea plant after subsequent field trials.

Keywords: Fungicides, Grey Blight, Pestalotiopsis, Trichoderma

INTRODUCTION

Grey blight caused by *Pestalotiopsis* spp, perceived generally as weak and opportunistic pathogen, is one of the most significant disease of *Camellia sinensis* (L.) O. Kuntze (Sanjay *et al.,* 2008). This species-rich asexual genus of coelomycetous fungi is heterogenous in nature and has more than 200 described species (Keith *et al.,* 2006b; Maharachchikumbura et al*.,* 2014). Genus *Pestalotiopsis* is cosmopolitan in nature and its species infect several economically important plants like Mango (*Pestalotiopsis mangiferae*), Coconut (*P. palmarum*), Rice (*P. versicolor*) and Tea (*P. theae*) and many other crops (Joshi *et al.,* 2009). In tea, several species of *Pestalotiopsis* have been reported of being associated with *Camellia sinensis*. *Pestalotiopsis theae* and *Pestalotiopsis longiseta* are considered to be economically significant species that have been reported in major tea-growing countries all over the world (Agnihothrudu, 1964 as cited in Joshi *et al.,* 2009). The species are differentiated primarily based on conidial characteristics such as size, septation, pigmentation, and presence or absence of appendages (Keith *et al.,* 2006). Conidial morphology is the widely used taxonomic character for the genus *Pestalotiopsis.*

Pestalotiopsis spp. invade tea plant through stomata, lenticels, hydathodes and mostly through mechanical wounds (Agrios, 2005) and this stress and wound eventually lead to the disease development by *Pestalotiopsis* (Hopkins and Mcquilken 2000; Madar *et al.,* 1991). In tea particularly, symptoms of infection by different species of *Pestalotiopsis* are seen after the foliage are injured by stress (Hopkins and Mcquilken 2000; Rivera and Wright 2007). *Pestalotiopsis* spp. primarily infect plant under stress and are also recognized as opportunistic and weak pathogen (Coyier & Roane, 1986). Initially, small, oval, pale yellow-green spots first appear on leaves which as the spots grow, turn brown or gray. Symptoms in plants infected with grey blight include appearance of circular or irregular shaped grey patches and lesions on the leaves. Then concentric rings in spots with scattered, tiny black dots (fruiting bodies) become visible and eventually dried tissue fall, leading to defoliation (Keith *et al.,* 2006).The tiny, black spots on the lesions contain the fungal spores which get transported with rain splashes from one plant or site of infection to another. When spores land on a leaf, they germinate to start a new leaf spot or a latent infection (Keith *et al.,* 2006).

Pestalotiopsis species since long have been identified as endophytes, but they could also survive as saprobe since they are able to switch their nutritional mode (Hu *et al.,* 2007) and have been isolated from dead leaves, bark and twigs (Maharachchikumbura *et al.,* 2013) which further complicates its management. The disease mainly infects maintenance leaves of tea, mature leaves and foliage which ensure nourishment to young shoots and tender foliage but bare stalk and young shoots are also affected which ultimately results in huge crop loss (Pallavi *et al.,* 2012; Joshi *et al.,* 2009; Horikawa, 1986).

Grey leaf blight in tea has been reported causing up to a 10-20% loss in production (Shin *et al.,* 2000; Keith *et al.,* 2006) while 17% production loss due to grey leaf blight have been recorded in Southern India (Joshi *et al.,* 2009). Losses by these diseases are devastating to farmers and when the impact on marginal farmers are taken in, the effect worsens. The primary strategy in controlling grey blight is fungicide application (Sanjay *et al.,* 2008). Fungicides like Mancozeb, Copper oxychloride, Carbendazim, Hexaconazole, Tridemorph, Azoxystrobin, Triflumizole, Bitertanol and Copper hydroxide are used in controlling this disease (Sanjay *et al.,* 2008; Kumhar *et al.,* 2016). But more work has to be done in dictating appropriate formulation, thereby preventing over and under application of fungicides. Fungicides like Thiophanate-methyl, Fluazinam, Chlorothalonil, Bitertanol, Copper hydroxide, Mancozeb, Azoxystrobin, Difenoconazole have been tested against grey blight and reduction in disease incidence has been between 85.7% to 90% but the interval period between harvesting, pruning, shearing and fungicide application plays a great role and should be as lower as possible for higher efficacy (Shin *et al.,* 2000). Besides these, modification in harvesting is also practiced in lessening the devastation caused by pathogen. Disease incidence is found least on fields with hand plucked harvesting and highest in continuously harvested plots. Practice of continuous hard shearing results weak host plant resistance (Sanjay *et al.,* 2008). Extensive use of machines in harvesting, shearing, leaves open cuts which allows easy pathogen entry. Grey blight disease earlier was managed manually by removing the infected leaves and burning them in the field, since it did not warrant a separate fungicide spraying *(Sanjay et al.,* 2008). Sanjay *et al.* (2008) reported that for grey blight, fungicides provided significant disease protection to the plants when compared with bio-control agents. On the other hand, experiment conducted by Kishor *et al.* (2016) reported *Trichoderma* isolates' performance on effectively controlling this pathogen and concluded that integrated approach in pathogen management would be more *fruitful* if

compatibility of BCA with fungicides were looked into. For long-term and environmentally friendly management of this disease, finding and deploying resistant varieties, specifically for organic tea production, is highly desirable (Akbar *et al.,* 2017). Growers may be able to minimize infection by avoiding physical plant damage and by growing plants to avoid stress. High temperatures and relative humidity also favors symptom development.

Although fungicides have been applied for protection for a very long time, over application associated with environmental toxicity is evident in tea crop. Fungicides share a major chunk with 49% of the total usage, followed by insecticide (33%), herbicide (14%) and then others (4%) among pesticides used in Nepal indicating rise in increased use of pesticides. Several studies have shown reports of unawareness about negative impacts of pesticide overuse (Kalauni & Joshi, 2019). Pesticide application has been reported higher in tea farms, moreover in CTC farms in the tropics (FAO, 2014). Sharma et al. (2013) have reported higher (2,100 g, a.i./ha) pesticide application rate in tea farms, some 15 times, more than the national average rate of 142 g, a.i./ha. Use of excessive pesticides in tea, or dependence on it will lead to more problems of poisoning, environmental hazard, residual effect and resistance development. Hence there is a need for a rationalized use of fungicides, determination and integration of effective alternative approaches. This study was conducted to evaluate comparative efficiency of two bio-control agents viz *Trichoderma harzianum* and *Trichoderma viride* and five chemical fungicides at different formulation to assess their rationality against *Pestalotiopsis theae*. This study plans to provide data of the bio-control alternative to the conventional control measure to work upon in further field trials at a much larger scale.

MATERIALS AND METHODS

Isolation and determination of pathogen

The experiment was conducted in Plant Pathology Laboratory of Agricultural Research Station (ARS), Pakhribas. The tea plantation site at ARS was surveyed to identify foliar diseases in tea. Grey blight was profusely observed in Pasang Lamhu variety. Diseased leaf sample with characteristic grey blight symptoms were collected. Leaf sample were cut into small pieces of about 2mm size and sterilized in 1% NaOCI for 30 seconds and then subsequently washed with sterile distilled water 3 to 4 times. The sterilized section was teased and under microscopic examination, pathogen were identified as *Pestalotiopsis theae* as identified by Maharachchikumbura, Chukeatirote & Hyde (2013) with fusiform shaped dark brown conidia, slightly constricted at septa, 4-septate, and 3-4 apical appendages from conidia and conidiophore in clusters were observed in abundance. The sterilized cut sections were then placed in moisture chamber and incubated for 3 days at 26±1° Celsius. After 3 days, profuse fruiting in cut section was observed with characteristic morphological character of grey blight. After determination, the sections were asceptically transferred to petri plates with PDA media on working surface of laminar air flow and incubated at 27±1 °C. After 2 subsequent subculturing, pure culture of *Pestalotiopsis theae* was obtained and maintained to conduct rest of the work.

In-vitro Efficacy of Commercial Fungicides

For the in vitro efficacy test, 5 commercially available fungicide; single and binary mixture of fungicides were selected. PDA media was prepared and autoclaved at 121°C for

20 minutes at 15 lbs pressure and a 1000 ppm of fungicidal stock solution was prepared which after calculation, was used to prepare 50ppm, 100ppm, 200ppm, and 500 ppm of poisoned media. The molten media was poured in petri dish under laminar air flow and left to solidify. For the poisoned media growth, actively growing 6 mm mycelial section of *P. theae* was placed at the center of the media plate. Each treatment was replicated 3 times with one control placed for each treatment.

Table 1. Selected commercial fungicides

	Copper Oxychloride 50% WP
	Carbendazim 50% WP
	Metalaxyl 8% WP + Mancozeb 64% WP
	Carboxin 37.5% WS + Thiram 37.5% WS
	Hexaconazole 5% EC

*WP- Wettable Powder, WS- Water dispersible powder, EC- Emulsifiable Concentrate

Bioefficacy of *Trichoderma viride* and *Trichoderma harzianum*

Trichoderma viride and *Trichoderma harzianum* were tested for their biocontrol potent against *Pestalotiopsis theae.* For dual culture test, a sterilized cork borer was used to cut an actively growing 6mm mycelial section of 8 days old *P. theae* and placed about a cm away from the edge of a petri dish, and at its opposite equidistant position 6mm section of actively growing mycelial section of 8 days old *T. harzianum* was placed. For both *P. theae* and *T. viride* active sections of 10 days old culture were cut and placed. Growth of both colony was measured radially and diagonally.

Research Methodology and Research Design

The experiment was conducted using a Completely Randomized Design (CRD), with each treatment allocated randomly and replicated 3 times with a control placed along with every treatment. Growth observations were recorded and inhibition percentage of fungal growth was calculated using the following formula (Vincent, 1947):

Where,

$$\text{Percent growth inhibition } (\%) = \frac{(C - T)}{C} x100$$

C= colony growth in control plate

T = colony growth in treated plate

Data recording and analysis

The recorded data were fitted and analysed using Genstat software 15.1.0.8035 version and MS excel. The analyzed data are presented in different graphs and in tabular form.

RESULTS AND DISCUSSION

Invitro Efficacy of Fungicides against *Pestalotiopsis theae*

Five fungicides at 4 different concentrations were tested against *Pestalotiopsis theae* using poisoned food technique.

Table 2. Efficacy of Fungicides at different concentration on growth of *Pestalotiopsis theae*

Fungicides	Mean colony growth (cm) of *Pestalotiopsis theae* at different concentration (ppm)			
	50	100	200	500
Copper Oxychloride	5.150b	4.300c	-	2.300e
Carbendazim	1.250fg	1.250f	1.300f g	1.150gh
Metalaxyl + Mancozeb	2.833d	2.600de	2.483de	0.000j
Carboxin + Thiram	0.700hi	0.600i	0.000i	0.000hi
Hexaconazole	0.000hi	0.000hi	0.000hi	0.000hi
Control (without fungicide)	8.0000a			
Grand mean	1.696			
F value	173.84			
P value	<0.001			
CV%	16.4%			
LSD	0.4578			
SE(m) ±	0.1602			

* Values are the mean of three replications. Different letter following means indicate significant difference at 0.05 level of significance.

The different letters in table 2 with numbers indicate that all five fungicides significantly inhibited mycelial growth of *Pestalotiopsis theae* in comparison to control. Among all treatments, Hexaconazole at all concentration exhibited complete (100%) inhibition in pathogenic growth. Besides this, complete (100%) inhibition was also observed at 200 ppm and 500 ppm of Carboxin + Thiram along with Metalaxyl + Mancozeb at 500 ppm. This complete inhibition was followed by 50 ppm and 100 ppm of Carboxin + Thiram with over 90% inhibition in mycelial growth.

In case of Metalaxyl + Mancozeb, a slight and uniform increase in inhibition percentage from 64.58% at 50 ppm to 67.5% at 100 ppm and then a rise to 68.96% at 200 ppm was recorded. There was reduction in pathogenic colony growth with increase in concentration. The growth in treated plates showed significant effect ($p<0.001$) when compared with growth in control plates.

In case of Carbendazim, the growth inhibition of the pathogen was also significant. The inhibition percentage did not fluctuate sharply and remained above 83% at all concentration. The growth reduction increased slightly from 84.37% at 50 ppm and 100 ppm to 85.62% at 500 ppm.

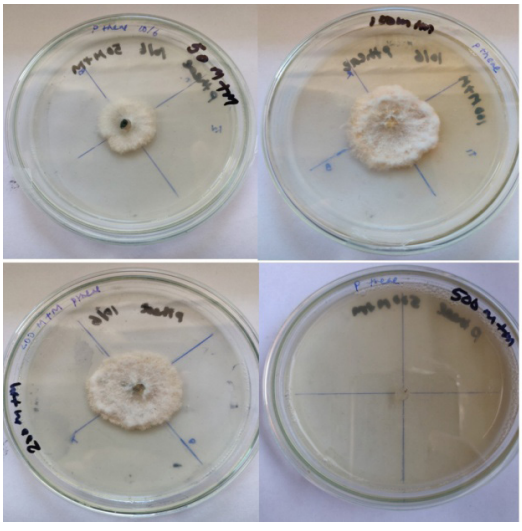
The least inhibition in pathogenic growth was observed in Copper Oxychloride at 50 ppm with around 35% growth reduction. Then, with increase in the fungicidal concentration, the inhibitory percentage increased to 71.25% at 500 ppm. While maximum growth of *P. theae* was observed in control plate, all treated plates provided significant reduction in growth.

Table 3. Growth inhibition percentage of different fungicidal concentration against *Pestalotiopsis theae*

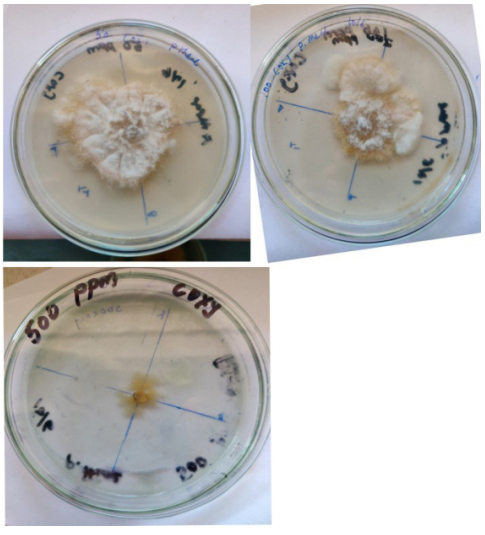
Fungicides	Growth inhibition percentage at different fungicidal concentration (ppm)				Mean growth inhibition percentage (%)
	50	100	200	500	
Copper Oxychloride	35.625	46.25	-	71.25	50.041
Carbendazim	84.37	84.37	83.75	85.62	84.52
Metalaxyl + Mancozeb	64.58	67.5	68.96	100	75.26
Carboxin + Thiram	91.25	92.5	100	100	95.83
Hexaconazole	100	100	100	100	100
Control (without fungicide)	0				0

Among the fungicides tested, Hexaconazole proved most effective with 100% growth restriction regardless of the concentration. Systemic fungicide like Hexaconazole, Carboxin, Metalaxyl have a narrow spectrum and inhibit metabolic processes and most effectively work as Ergosterol biosynthesis inhibitor due to which it reduces fungal sporulation, spore germination and its viability (Li *et al.,* 2013). Copper Oxychloride on the other hand exhibited relatively lower inhibition but the growth control increased with increase in concentration from 50 ppm to 500 ppm. Copper based fungicides denature cellular proteins at nonspecific site and disrupt function of protein and enzymes and result in cell damage and membrane leakage (Husak, 2015). The lower inhibition by Copper Oxychloride could be the result of the resistance developed by *P. theae* due to the prolonged and over usage of Copper based fungicides over the years. Hence at lower concentration, the application of these long used fungicide might be redundant. A similar study by Shin *et al.* (2000) emboldens the conclusion from this study where *Pestalotiopsis* isolates were found insensitive to copper based fungicides. While in the case of Carbendazim, where the growth reduction percentage in at all 4 concentrations did not change significantly from each other which meant that even with increase from 50 ppm to 500 ppm the difference in the growth of *P. theae* was uniform. Hence over usage would be rather an addition to the already high environmental residue. This also holds true in the case of Hexaconazole where complete inhibition at 50 ppm meant that the pathogen growth control was achieved even at lower concentration.

The mycelial growth inhibition percentage in treated plates ranged from 35 % to complete inhibition at 100%. In case of binary mixture of contact and systemic fungicides, Carboxin + Thiram provided higher inhibition compared to the combination of Metalaxyl + Mancozeb. But the colony growth of pathogen reduced significantly with increase in concentration in both of these treatments. The results are in line with findings of study by Anitha *et al.* (2011) and Sanjay *et al.* (2008) where combination of contact and systemic fungicides provided significant reduction in grey blight incidence in tea.



Metalaxyl + Mancozeb



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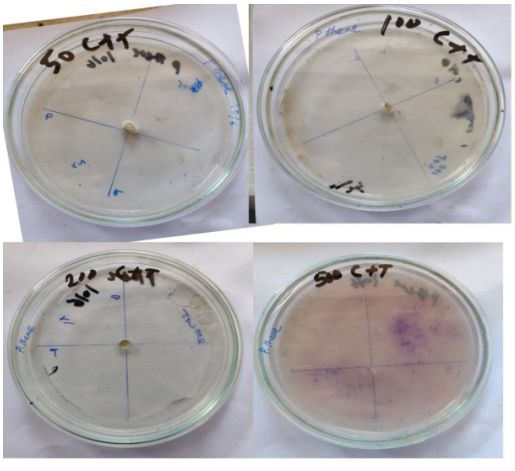
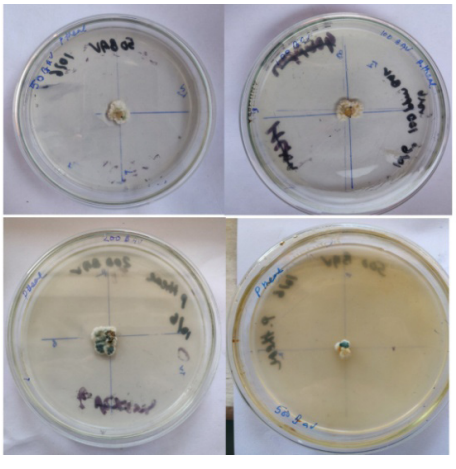
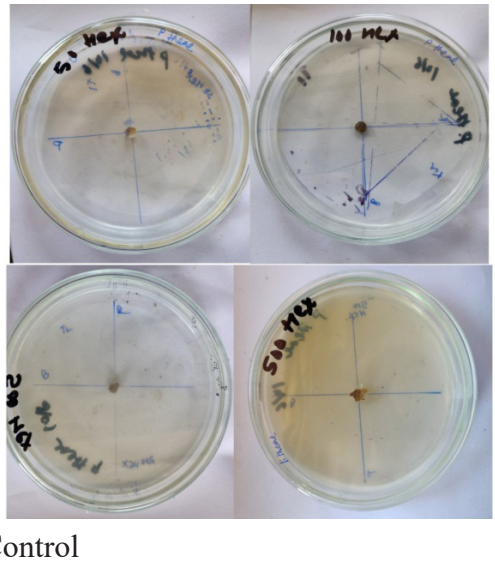


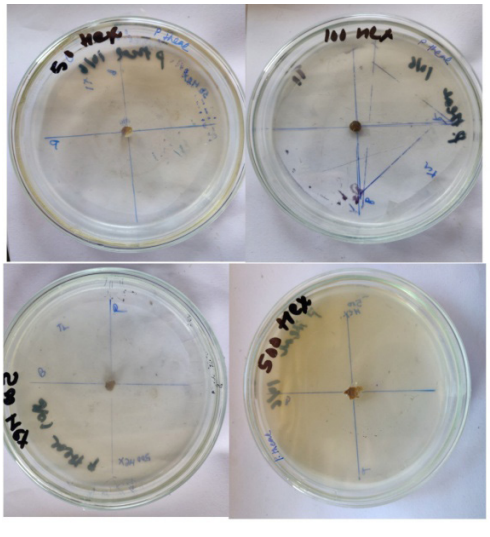
Carboxin + Thiram



Carbendazim



Control



Hexaconazole

Figure 2. Effect of Fungicidal concentration on mycelial growth of *Pestalotiopsis theae*

The overall results are in line with the study by Kumhar *et al.* (2016), where Hexaconazole and mixture of fungicides like Mancozeb, Carbendazim provided significant growth inhibition of over 70% against *P. theae*. Sanjay *et al.* (2008) reported 6.6% of grey leaf blight incidence with Carbendazim application which was most effective in protection against *P. theae*. The results are also in congruence with findings from Sanjay *et al.* (2008) in grey blight where pathogen suppression was better in systemic fungicides than in contact fungicides.

Dual culture assay of *Trichoderma* spp and *Pestalotiopsis theae*

Trichoderma viride and *Trichoderma harzianum* were tested for their effectivity in growth suppression of *Pestalotiopsis theae*. The dual plate assay between *T. harzianum* and *P. theae* resulted in 62.32% growth inhibition of *P. theae*. After 11 days of dual culturing both fungi, mycelial growth of *T. harzianum* covered that of *P. theae* completely but growth reduction was evident prior to the direct contact.

Similarly antagonistic activity of *T. viride* was observed against *P. theae*. *T. viride* exhibited 66.64% growth reduction on colony growth of *P. theae* on day 8.

Table 4. Mean radial growth of *Pestalotiopsis theae* against *Trichoderma harzianum*

Treatment	Mean radial growth of *P. theae* (cm)	Inhibition percentage of mycelial growth
Trichoderma harzianum	1.733	62.32%
Control	4.6	0%
CV%	3.4	
Grand mean	3.167	
SE(m) ±	0.0624	

Table 5. Mean radial growth of *Pestalotiopsis theae* against *Trichoderma viride*

Treatment	Mean radial growth *P theae* (cm)	Inhibition percentage of mycelial growth
Trichoderma viride	0.567	66.64%
Control	1.700	0%
CV%	7.2	
Grand mean	1.133	
SE(m) ±	0.0471	

Both of the bio-control agents provided significant reduction in growth of *P. theae* owing to their characteristic antagonism. *Trichoderma* spp. opt several pathways in activating defense mechanism against pathogen. These fungi enhance plant growth and antagonize plant pathogen by using mechanisms like mycoparasitism, antibiosis, modification of the environmental conditions, and stimulating plant growth and plant defense mechanisms. Beside many other things *Trichoderma* also compete for nutrient and space against pathogen. The findings are synchronous with the result by Kumhar *et al.* (2016) who reported significant inhibition of 61.5% by *T. viride* and 57% by *T. harzianum* against *Pestalotiopsis theae*. The increase in usage of *Trichoderma* isolates as bio-control agent is credit to its multifaceted advantages. They produce toxins, lytic enzymes, volatile compounds that target and kill pathogen (Gorai *et al.,* 2020). One of the recognized pathway in pathogen antagonism is

production of cell wall lytic enzymes like chitinase, glucanase, protease and metabolites like Harzianic acid, Alamethicins, Tricholin, Peptaibols etc. when in contact with a pathogen (Benítez *et al.,* 2004; Vey *et al.,* 2009). The results are also coherent with findings from study by Harikamal *et al.* (2015) which found maximum inhibition of 72.4% against mycelial growth of *P. theae* which concurs with the findings of this study.

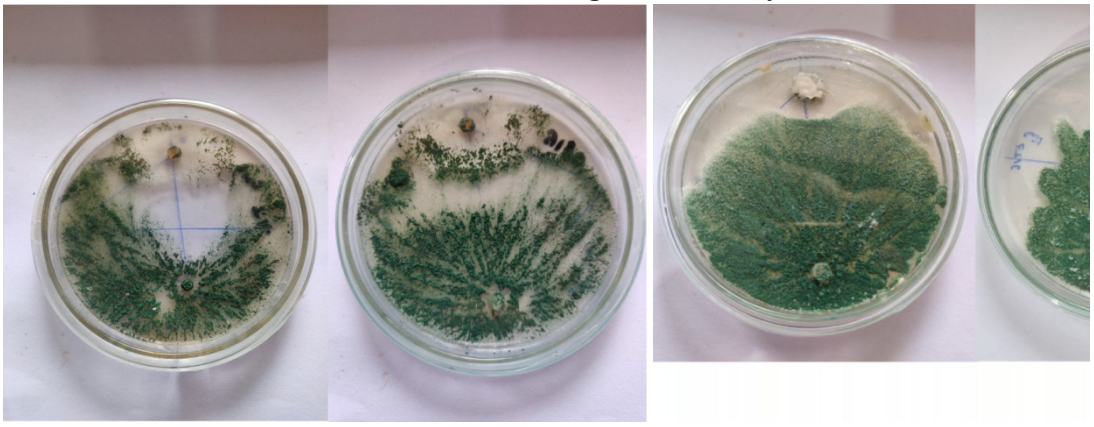


Figure 3. Dual culture of *Trichoderma harzianum* and *Pestalotiopsis theae* P. theae growth in control plate

Figure 4. Dual culture of *Trichoderma viride* and *Pestalotiopsis theae*

CONCLUSION

Grey leaf blight is a widespread disease, inflicting heavy loss on tea plant especially in plants under stress and older leaves that nourish new flush. *Trichoderma harzianum* and *Trichoderma viride* were able to suppress mycelial growth of *Pestalotiopsis theae* in this current study with significant difference. Similarly, all five commercial fungicides, at all concentration provided significant reduction in growth of *Pestalotiopsis theae* in comparison to control. Complete inhibition in mycelial growth was recorded only with chemical fungicides. As fungicidal concentration increased, mycelial growth of pathogen reduced. This result indicates the possibility and potentiality of integrated management approach with bio-control agents and fungicides in managing grey leaf blight in tea. There is a need of further field trials to establish the results of this study.

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