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Antifungal and Morphological Assay of Selective *Trichoderma* Isolates Against Soil Borne Plant Pathogenic Fungi

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ABSTRACT: Soil borne plant pathogens cause potential yield loss in every year all over the world. Antifungal *Trichoderma* isolates can control soil borne plant pathogenic fungi. Soil samples were collected from different agricultural fields to find out effective *Trichoderma* isolates. Their antifungal and morphological characteristics were studied. Radial mycelial growths of the isolates varied from 29.91 to 90.00 mm in vitro assay. On the basis of shape, growth habit, colony color, compactness, spore density isolates were categorized in different groups. Different selected temperature such as: 20^{o}, 25^{o}, 30^{o} and 35^{o} C were maintained in incubators to observe the growth habit of different *Trichoderma* isolates. Optimum growth of *Trichoderma* isolates was recorded 25 to 30ºC. Antifungal activities of all *Trichoderma* isolates were placed against seven fungal mycoflora viz. *Penicillium sp, Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani, Aspergillus flavus, Colletotrichum gloeosporiodes and Phomopsis vexans* by duel culture method. All isolates of *Trichoderma* showed wide variation in growth inhibition against these pathogens. Among all of the *Trichoderma* isolates, ST_5 was strongly inhibited the growth and pathogenicity of the pathogenic fungi.

KEYWORDS: Antifungal, Bio-control agent, Soil-borne fungus *Trichoderma* spp.

1 INTRODUCTION

Soil borne pathogens have wide host range and able to persist for long period in soil by forming resistant structures [1]-[2]. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods [3]. The antagonistic micro-organisms successfully reduce the crop damage caused by different plant pathogenic fungi [4]. *Trichoderma* spp are found in almost all tropical and temperate soil. Several strains have been found to be effective as bio control agents of various nematodes, soil-borne plant pathogenic fungi such as *Rhizoctonia, Aspergillus, Pythium, Phytophthora, Fusarium oxysporum, Sclerotinia sclerotiorum* etc. under greenhouse and field conditions [5]-[11]. The efficacy of this depends largely on the physical, chemical and biological conditions of soil. The suppression of disease by *Trichoderma* is based on hyper parasitism, antibiosis, induced resistance in the host plant and competition for nutrients and space [12]. However, chemical control of soil borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms. Pesticides and organic compounds are not degrading completely and leave toxic residues in food chain [13]. Farmers are trying to overcome this problem through different cultural practices and use of chemical fungicides. But the control of soil borne pathogens with chemicals is very expensive. In addition, unwise use of chemicals in agriculture causes environment pollution and health hazards, destroying the natural balance and beneficial micro-flora of the soil [14]. Moreover, consumers are becoming increasingly concerned about chemical pollution of the environment and pesticide residues in food and farmers more often face with development of pathogen's resistance to chemical fungicides. Therefore, there is a need for development of efficient alternative measures to combat the diseases and inoculums build up in soil [15].

In such views, the present experiment was undertaken to isolate and identify the *Trichoderma* spp from the soil having potential antifungal effect against wide range of soil borne plant pathogens.

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2 MATERIALS AND METHODS

2.1 ISOLATION OF *TRICHODERMA* SPP

The soil samples were collected from the different locations of Bangladesh (Table 1). *Trichoderma spp.* were isolated from soil by dilution plate technique [16]. Individual morphological features were observed for identification. Optical observation on petri dishes and micro-morphological studies in slide culture were implemented for identification of *Trichoderma* species. The well-developed pure cultures were sub-cultured to PDA plate and transferred to PDA slants for preservation. The fully grown *Trichoderma* in slants were preserved in the refrigerator at 5ºC for further use (Figure 1).

2.2 COLLECTION OF SOIL BORNE PLANT PATHOGENS

The highly virulent pathogen *Penicillium sp, Fusarium oxysporum, Sclerotium rolfsii, Rhizoctonia solani, Aspergillus flavus, Colletotrichum sp* and *Phomopsis vexans* were collected from IPM Laboratory, Bangladesh Agricultural University, Mymensingh; Plant Disease Clinic, Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh; Bangladesh Rice Research Institute, Joydebpur, Gazipur. The pure culture of pathogen was preserved in PDA slants at 5 ± 1^{0}C as stock culture (Figure 2).

2.3 DETERMINATION OF ANTIFUNGAL PROPERTIES OF *TRICHODERMA* ISOLATES THROUGH DUAL CULTURE METHOD

The *Trichoderma* isolates were evaluated against previously mentioned soil borne fungi by dual culture technique [17]. A 6 mm diameter mycelial disc from the margin of each 7 days-old culture of *Trichoderma* isolates and the soil borne pathogens was place on the opposite at equal distance from the periphery. The experimental design used was a completely randomized with three Petri dishes for each isolates. In control plates (without *Trichoderma*), a sterile agar disc was placed. All plates were incubated at 28 ± 1^{o}C. After 6 days incubation period, radial growth of pathogen isolates was measured and percent inhibition of average radial growth was calculated following the formula [18].

$L = [(C – T)/C] \times 100$

Where, L is inhibition of radial mycelial growth; C is radial growth measurement of the pathogen in control; T is radial growth of the pathogen in the presence of *Trichoderma* isolate.

2.4 MORPHOLOGICAL CHARACTERISTICS OF *TRICHODERMA* ISOLATES

Radial mycelial growth of the isolates were studied following the method of [19]. For visual observation, the isolates were grown on PDA agar for 3-6 days. Morphological parameters were observed and measured starting from 48 hours of inoculation. After 24, 48 and 72 hours of inoculation, the radial mycelial growth was measured as the mean of two perpendicular diameters and mean of three replications were taken as growth of each isolate. The mode of shape, color, growth habit and compactness for each isolate were examined every day.

2.5 MEASUREMENT OF SPORE DENSITY

In order to determine spore density, ten days old conidial suspension of *Trichoderma* isolates were taken in a beaker from the media and stirring. The volume of the beaker with conidial suspension was made about 500ml with sterile water and 1 drop Tween-20 was added to it and stirred to disperse well. From this solution 1 drop of suspension was taken on the center of Haemacytometer and a cover slip was placed on it. Finally spores were counted under microscopic power of 40X (Figure 3).

Putting the average number of spore per unit cell in the following formula, the number of spore per 1 ml was determined.

$$\text{Number of spores per cubic mm suspension} = \frac{\text{Number of spores counted X dilution}}{\text{Number of smallest square counted}} X\,4000$$

2.6 EFFECT OF TEMPERATURE

Trichoderma isolates allowed growing at different selected temperature such as: 20^o, 25º, 30º and 35^{o}C on PDA media (3 replication). After 3 days incubation of *Trichoderma* isolates, radial mycelial growth were checked for each petri dish.

2.7 ANALYSIS OF DATA

Data collected during experimental period were tabulated and analyzed through a standard computer package statistical procedure MSTAT- C [20].

3 RESULTS AND DISCUSSION

3.1 ANTIFUNGAL EFFECT OF *TRICHODERMA* SPP. ON PATHOGENIC FUNGI

In this study, ten (10) *Trichoderma* isolates were isolated from soil samples of different locations using dilution plate technique and named as ST_1, ST_2, ST_3, ST_4, ST_5, ST_6, ST_7, ST_8, ST_9, and ST_{10}. Seven plant pathogenic fungi such as *Penicillium* sp, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Aspergillus flavus*, *Colletotrichum gloeosporiodes* and *Phomopsis vexans* were show significant inhibitory effect by dual culture method (Table 2). Radial mycelial growth of *Penicillium sp* against all Trichoderma isolates were measured. At 6 days after inoculation (DAI), the lowest mycelial growth (0.16 mm) was found in ST_8 which was followed by ST_6 (0.46 mm). *Trichoderma* isolate ST_8 inhibited at maximum percent fungal growth of *Penicillium* sp (99.58%) followed by ST_6 (98.84%) A similar findings also observed [21]. All isolates of *Trichoderma* showed wide variation in growth of *Fusarium oxysporum* that varied from 0.33 to 9.23 mm at 6 DAI. The highest percent inhibition (99.58%) was found in ST_5 followed by ST_8 (88.44%). Dubey [22] found that mycelial growth of *F. oxysporum* was inhibited with *T. harzianum*. Growth inhibition of *Sclerotium rolfsii* by *Trichoderma* isolates varied from 59.61 to 99.70 % at 6 DAI. The highest percent inhibition (99.70%) was found in ST_5 followed by ST_8 (95.33%). Similar findings had been also reported [2]. They reported 67.91% inhibition of *S. rolfsii* with *T. harzianum,* while [23] found maximum of 88.80% inhibition of *S. rolfsii* by *Trichoderma harzianum*. Antifungal activity of *Trichoderma* isolates against *Rhizoctonia solani* showed wide variation in growth inhibition of *Rhizoctonia solani* that varied from 75.29 to 99.85 % at 6 DAI. The highest percent inhibition (99.85%) was found in ST_6 followed by ST_2 (99.59%). Maximum 88% inhibition of *Rhizoctonia solani* by *T. harzianum* also recorded [23]. These report in agreement with the results of the present study. Growth inhibition of *Aspergillus flavus* varied from 61.65 to 100 % at 6 DAI. The highest percent inhibition (100%) was found in ST_6 and ST_8 followed by ST_4 (91.33%). In case of *Colletotrichum gloeosporiodes* and *Phomopsis vexans*, ST_5 and ST_3 showed highest inhibition 91.87 and 100 percent over control, respectively, followed by ST_6 showing 91.46 and 99.82 percent, respectively. Similar findings had also been reported [24] – [25].

3.2 COMPARISON OF ANTIFUNGAL ACTIVITY OF THE BEST THREE ISOLATES (ST_5, ST_6 AND ST_8)

Trichoderma isolates ST_6 and ST_8 can't fully control *Sclerotium rolfsii* and *Colletotrichum gloeosporiodes* (Figure 4). However, the ST_5 can control significantly all the tested pathogenic fungi.

3.3 MORPHOLOGICAL CHARACTERIZATION OF *TRICHODERMA SPP*

Different isolates of *Trichoderma* spp. distinctly differed on their cultural and morphological properties. Mycelial growth rate of different isolates varied considerably up to 72 hours (3 days). Linear/apical growth of mycelia on PDA plates at 28±1ºC ranged from 29.91 mm to 90.00 mm (Table 3). Based on color, growth habit and colony consistency on PDA medium were divided into several groups. Based on color, isolates were divided into five groups. Results indicated that isolate ST_3 had dark green color which was distinctly different from other isolates. Three isolates ST_1, ST_9, and ST_{10} showed green colony. ST_4, and ST_8 isolates were light green color. Out of 10 isolates, only ST_5 was yellowish green. Rest of the *Trichoderma* isolates ST_2, ST_6 and ST_7 were whitish green. On the basis of growth, isolates were divided into three categories such as fast, medium and slow. Among these all isolates had fast growth, some had medium growth and few ST_4, ST_7 performed slow growth. Similarly, on the basis of colony consistency, the isolates were categorized into three groups such as very compact, compact and loose. Few isolates were very compact such as ST_3, ST_7, ST_8, and ST_{10}; most of the isolates showed compact appearance and only few ST_2, ST_9 were loose (Table 4) [26].

Spore density among different *Trichoderma* isolates were determined. Highest spore density was recorded among isolates of dark green and yellowish green color. Whereas lowest was found among the isolates of light green, green and whitish green color (Table 5). From this result, it is evident that Trichoderma isolates showed differences in spore production [27].

In this study, it was found that *Trichoderma* grew well at 25-30ºC rather than below 25ºC and above 35ºC, respectively (Figure 5) [28] - [29]. They found significant differences among the strains were noted between 20 and 30 °C.

Table 1. Name of Trichoderma isolates and their sources.

Sl. No.	*Trichoderma* isolates	Sources
1	ST_1	Mymensingh - 1
2	ST_2	Gazipur
3	ST_3	Faridpur - 1
4	ST_4	Jamalpur
5	ST_5	Sherpur
6	ST_6	Rangpur
7	ST_7	Netrokona
8	ST_8	Faridpur - 2
9	ST_9	Tangail
10	ST_{10}	Mymensingh -2

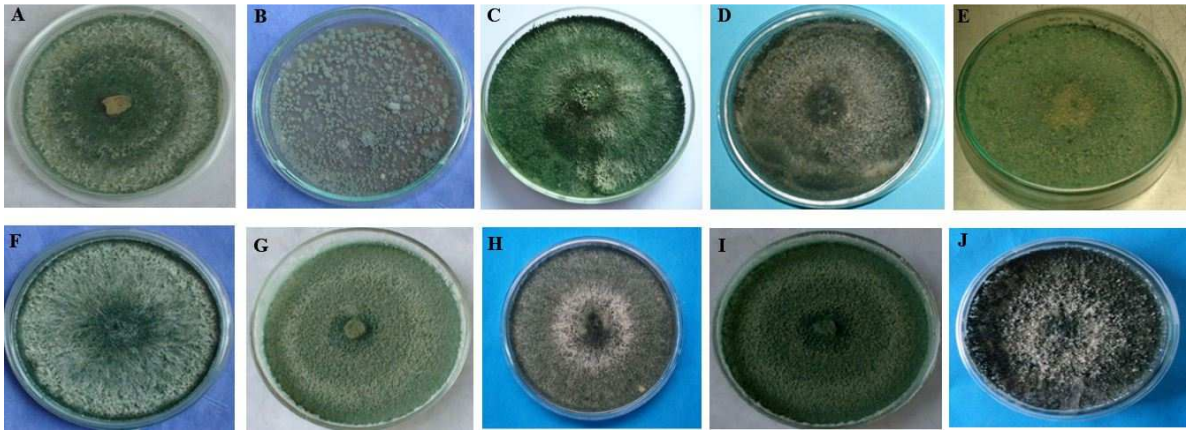

Fig. 1. Pure culture of Trichoderma isolates.

A: ST1, B: ST2, C: ST3, D: ST4, E: ST5, F: ST6, G: ST7, H: ST8, I: ST9, J: ST10.

Fig. 2. Pure Culture of Plant Pathogenic fungi.

A: Penicillium sp, B: Fusarium oxysporum, C: Sclerotium rolfsii, D: Rhizoctonia solani, E: Aspergillus flavus, F: Colletotrichum gloeosporiodes, G: Phomopsis vexans

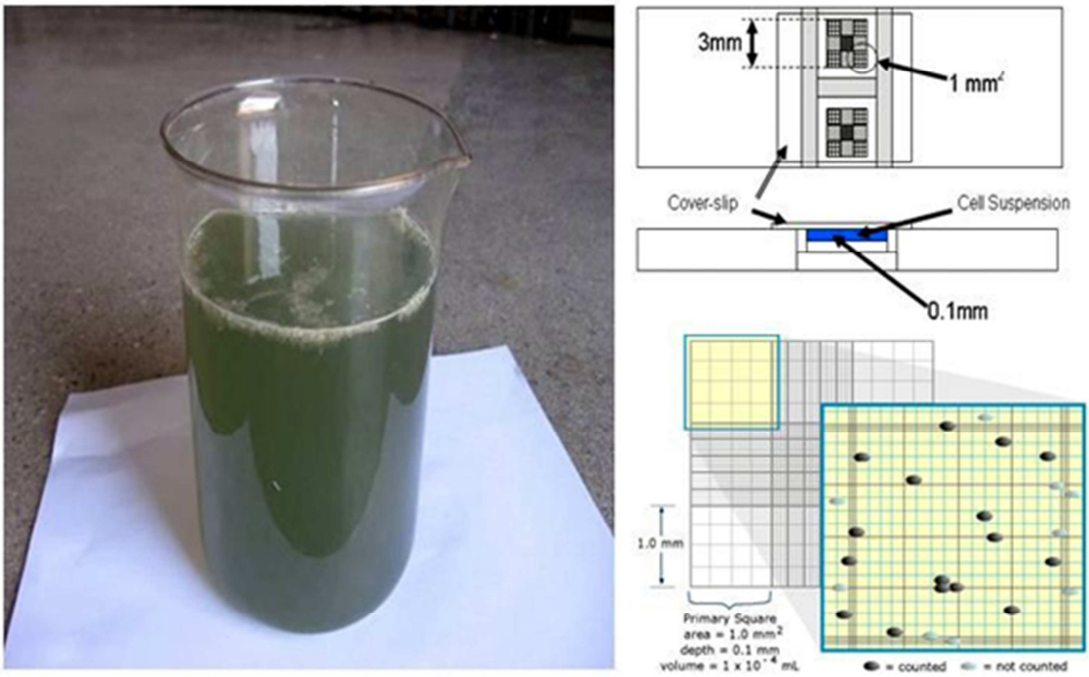

Fig. 3. Spore suspension and counting in Haemacytometer

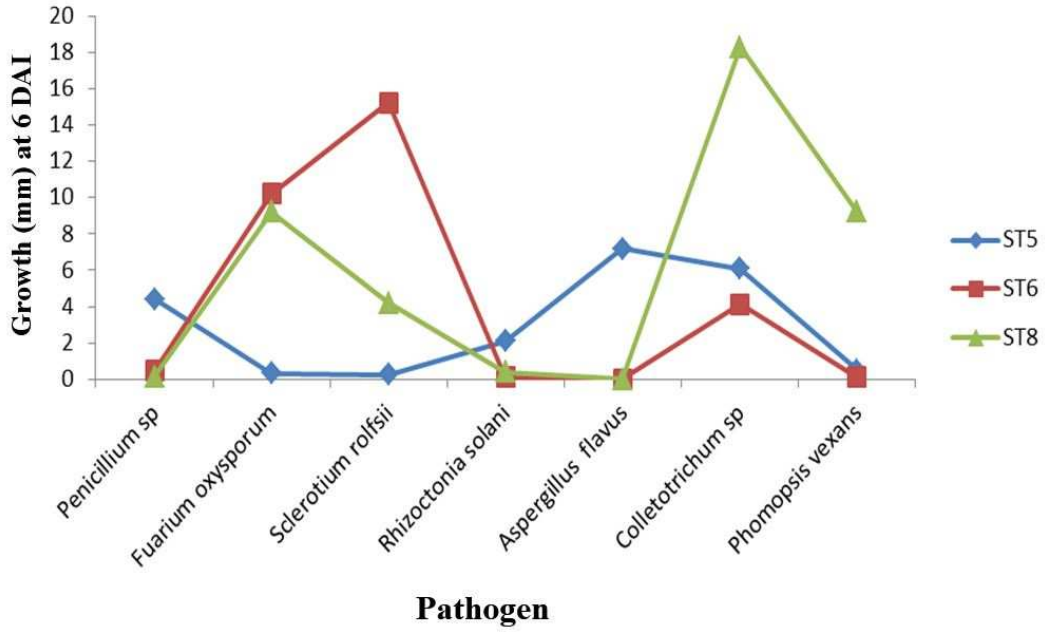

Fig. 4. Effect of selective Trichoderma isolates on the growth of different pathogenic fungi

Table 2. Effect of different Trichoderma isolates against Plant pathogenic fungi by dual culture (6 DAI)

Trichoderma Isolates	Plant Pathogen																					
	Penicillium sp		*Fusarium oxysporum*		*Sclerotium rolfsii*		*Rhizoctonia solani*		*Aspergillus flavus*		*C. gloeosporiodes*		*Phomopsis vexans*									
	Mycelial Growth (mm)	% Inhibition	Mycelial Growth (mm)	% Inhibition	Mycelial Growth (mm)	% Inhibition	Mycelial Growth (mm)	% Inhibition	Mycelial Growth (mm)	% Inhibition	Mycelial Growth (mm)	% Inhibition	Mycelial Growth (mm)	% Inhibition								
ST1	2.10 h	94.78	19.26 c	75.89	20.26 f	77.52	4.10 e	95.44	12.23 d	80.01	33.10 b	55.92	4.33 f	94.23								
ST2	7.26 e	81.96	20.10 b	74.85	26.20 e	70.94	0.36 g	99.59	6.30 g	89.70	12.16 h	83.79	8.23 d	89.06								
ST3	8.16 d	79.73	12.16 e	84.77	4.23 i	95.30	14.30 d	84.11	9.26 e	84.85	13.36 g	82.20	0.00 g	100								
ST4	6.36 f	84.20	10.23 f	87.19	19.33 g	78.55	0.23 g	99.74	5.30 h	91.33	23.46 c	68.75	20.20 c	73.16								
ST5	4.43 g	89.00	0.33 h	99.58	0.26 j	99.70	2.10 f	97.66	7.20 f	88.23	6.10 i	91.87	0.50 g	99.33								
ST6	0.46 i	98.84	10.20 f	87.23	30.13 c	83.12	0.13 g	99.85	0.00 i	100	4.13 j	91.49	0.13 g	99.82								
ST7	15.10 b	62.53	18.26 d	77.14	36.50 b	59.61	22.23 b	75.29	22.30 c	63.56	15.56 f	79.27	21.33 b	71.65								
ST8	0.16 i	99.58	9.23 g	88.44	15.50 h	95.33	0.40 g	99.55	0.00 i	100	18.30 e	75.63	9.23 d	87.73								
ST9	10.23 c	74.60	19.63 bc	75.43	15.20 h	83.14	18.23 c	79.74	23.46 b	61.65	19.26 d	74.34	4.16 f	94.46								
ST10	15.20 b	62.28	20.13 b	74.81	28.13 d	89.85	0.13 g	99.85	23.10 b	62.25	12.26 h	83.66	6.33 e	91.58								
Control	40.30 a		79.93 a		90.16 a		90.00 a		61.20 a		75.10 a		75.26 a									
LSD	0.79		0.82		0.82		0.75		0.74		0.72		1.09									

DAI = Days After Inoculation

Table 3. Radial mycelial growth of 10 isolates of Trichoderma spp

Isolates Name	Mycelial diameter at 3 DAI (mm)	Mycelial diameter at 6 DAI (mm)
ST_1	86.62 d	90.00 a
ST_2	87.69 b	89.38 b
ST_3	62.58 e	86.69 c
ST_4	29.91 h	89.89 a
ST_5	89.67 a	90.00 a
ST_6	89.54 a	89.83 ab
ST_7	36.56 g	78.50 d
ST_8	89.39 a	90.00 a
ST_9	86.30 c	89.96 a
ST_{10}	44.00 f	85.86 c
LSD	0.616	0.479

Table 4. Colony characters of Trichoderma isolates

Isolates		General characteristics			
SL.No.	*Trichoderma* isolates	Shape	Color	Growth habit	Colony consistency
1.	ST_1	Regular	Green	Fast	Compact
2.	ST_2	Regular	Whitish green	Fast	Loose
3.	ST_3	Regular	Dark green	Medium	Very Compact
4.	ST_4	Regular	Light green	Slow	Compact
5.	ST_5	Regular	Yellowish green	Fast	Compact
6.	ST_6	Regular	Whitish green	Fast	Compact
7.	ST_7	Regular	Whitish Green	Slow	Very Compact
8.	ST_8	Regular	Light green	Fast	Very Compact
9.	ST_9	Regular	Green	Fast	Loose
10.	ST_{10}	Regular	Dark green	Medium	Very Compact

Table 5. Spore density of Trichoderma isolates

SL No.	*Trichoderma* isolates	Sporulation capacity (Number of spore/ml)
1.	ST_1	0.84×10^{10}
2.	ST_2	0.91×10^{10}
3.	ST_3	0.84×10^{10}
4.	ST_4	0.59×10^{10}
5.	ST_5	1.1×10^{10}
6.	ST_6	1.11×10^{10}
7.	ST_7	0.93×10^{10}
8.	ST_8	0.98×10^{10}
9.	ST_9	0.74×10^{10}
10.	ST_{10}	1.01×10^{10}

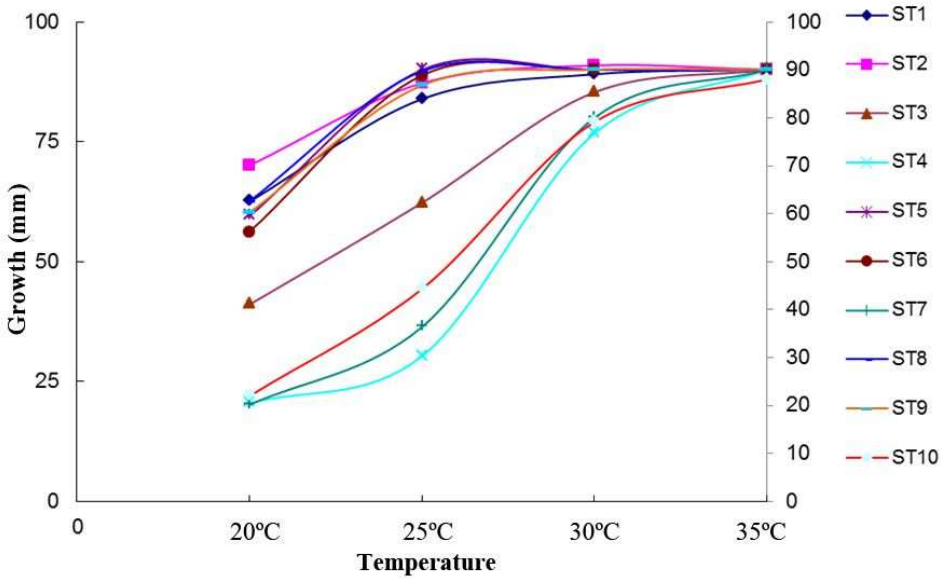

Fig. 5. Effect of temperature on the growth of Trichoderma isolates

4 CONCLUSION

This study was carried out to identify antifungal *Trichoderma* isolates from the soil rhizosphere to control soil borne plant pathogenic fungi. None of the isolates of *Trichoderma* showed complete growth inhibition towards tested seven pathogenic fungi but among all of the isolates, only ST_5 was found to strongly inhibit the growth of all pathogenic fungi under this experiment. So, further research is demanding on *Trichoderma* isolate ST_5 to get more details especially on its suitable substrates, antifungal and genomic information.

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