Research Journal of Biology, 2: 29 – 36 (2014)
www.researchjournalofbiology.weebly.com

RESEARCH JOURNAL OF BIOLOGY
E-ISSN: 2322-0066

RESEARCH ARTICLE Open Access

In vitro Screening for Antagonistic Potential of Seven Species of *Trichoderma* against Different Plant Pathogenic Fungi

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Abstract

The antagonistic potential of seven different species of *Trichoderma* isolated from the rhizosphere soils of tomato and chilli plants were evaluated in vitro against the most widely occurring soil inhabiting plant pathogens viz., *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina* and identify the *Trichoderma* strain with high antagonistic potential. All the isolates of *Trichoderma* species highly inhibited the growth of the three test pathogens by producing volatile and non-volatile inhibitors showing variability in antagonistic potential of different *Trichoderma* species against the different pathogens tested. Maximum growth inhibition of *Alternaria solani* (86.6% and 86.6%) and *F. oxysporum* (81.1% and 80.0%) was recorded by *T. virens* and *T. pseudokoningii* respectively, by producing volatile inhibitors. Whereas, growth inhibition of *A. solani* (86.6% and 83.3%) and *F. oxysporum* (82.2% and 78.9%) by *T. virens* and *T. pseudokoningii* respectively, was recorded by producing non-volatile inhibitors. However, *M. phaseolina* was comparatively less inhibited by all the species of *Trichoderma* by producing volatile and non-volatile compounds. *T. virens, T. pseudokoningii, T. atroviride* and *T. koningii* showed high antagonistic potential against *A. solani* and *Fusarium oxysporum* f. sp. *lycopersici*.

Key Words: Antagonism, chilli, tomato, *Trichoderma*, volatile and non-volatile inhibitors.
(Received: 23/02/2014; Accepted: 27/03/2014; Published: 15/04/2014)

Introduction

Diseases are the important biotic causes for low crop yield and poor quality seed. Pathogens being soil and seed borne, posses a great problem in managing the disease. Soil borne diseases are difficult to control and seed treatment with fungicides does not protect the crop for longer periods. Continuous use of the same fungicide against the same pathogen results in the development of fungicide resistant strains of the pathogen (Shanmugam and Varma, 1998; Kumar and Dubey, 2001; Mamgain *et al.*, 2013).

Moreover chemical measures may establish imbalances in the microbiological community unfavourable for the activity of beneficial organisms which otherwise improve the crop health. The demand for alternative to chemical control of plant pathogens has become stronger owing to the concerns about the safety and environmental aspects of chemicals. However, biological control offers the chance to improve crop production within the existing resources, besides avoiding the problem of pesticide resistance (Dekker, 1976; Khan *et al.*, 2014).

Species of the genus *Trichoderma* are ubiquitous in the environment and especially in the soil. Characterization for the antagonistic potential of *Trichoderma* species is the first step in utilizing the full potential of *Trichoderma* sp. for specific applications. *In vitro* screening of different pathogens is an effective and rapid method for identifying strains with antagonistic potential. *Trichoderma*, a filamentous soil borne mycoparasitic fungus, has been shown to be effective against many soil borne plant pathogens (Papavizas, 1985; Pan *et al.*, 2001; Jash and Pan, 2004) as they have more than one mechanism of action.

Therefore, the present study was conducted to evaluate the antagonistic potential of seven different *Trichoderma* species viz., *Trichoderma viride*, *T. harzianum*, *T. reesei*, *T. atroviride, T. pseudokoningii*, *T. koningii* and *T. virens*, in inhibiting the growth of some most widely occurring soil inhabiting plant pathogens viz., *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina* and identify *Trichoderma* strain with a high antagonistic potential.

Materials and methods

The isolation of seven different species of *Trichoderma* was made by soil dilution plate technique (Johnson and Curl, 1972) on modified *Trichoderma* Selective Medium (TSM) (Saha and Pan, 1997). The green coloured colonies were identified by slide culture technique and compared with taxonomic key of Rifai (1969) at genus and species level. The cultures of *Trichoderma* spp. were purified by single spore isolation technique, maintained on PDA slants and stored in the refrigerator at 4°C for further use.

The pathogens were isolated from diseased tomato and chilli plants showing symptoms of *Fusarium* wilt, early blight, charcoal rot and crown rot. These

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isolated pathogens were identified, purified and tested for pathogenicity (Guvvala *et al.*, 2013).

The hyperparasitic potential of seven *Trichoderma* species were screened *in vitro* against four test plant pathogens viz., *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina* by dual culture plate technique and production of volatile and non-volatile metabolites.

In vitro screening for fungal antagonists

Seven different species of *Trichoderma* were screened against test pathogens *in vitro*.

Efficacy of *Trichoderma* spp. on growth of the pathogens by dual-culture plate Method

For testing antagonism in dual culture method (Morton and Stroube, 1955) a mycelial disc (6mm) was cut from the margins of actively growing region of 5 day old cultures of *Trichoderma* species and inoculated at one end of the petri plates (1cm away from the edge of the plate) with sterilized potato dextrose agar (PDA) medium and simultaneously at the opposite end a mycelial disc (6mm) of the test pathogen. For each treatment three replicates were maintained and were incubated at 28±1^{o}C. Control plates were maintained for each pathogen. In dual cultures the *Trichoderma* isolates were categorized as effective based on their ability to overgrow and inhibit the growth of the pathogens by giving them a score as per modified Bell's scale (Bell *et al*., 1982), where R1= 100% overgrowth, R2= 75% overgrowth, R3= 50% overgrowth, R4= locked at the point of contact.

Hyphal interaction

Hyphal interactions between the antagonists and the test pathogens was studied from the dual culture plate technique. Mycelial mats were lifted gently with the help of the needle from the zone of interaction in the dual culture plates and placed in the drop of cotton blue on a microscopic slide. Mycelial mats were further transferred to a clean slide containing lactophenol and observed under the microscope.

Antibiosis

The effect of volatile and non-volatile compounds produced by the *Trichoderma* isolates, on the growth of the pathogen was studied as per the technique described by Dennis and Webster (1971a, b).

Effect of volatile metabolites

Production of volatile antibiotic study was done following the method of Dennis and Webster (1971b). Seven species of *Trichoderma* were inoculated in the center of the petri plates containing solidified sterilized PDA medium by placing 6mm disc (3d- old culture) from the margin of the actively growing region of *Trichoderma* sp. and incubated for 3 d at 28±1^{o}C. After that the top lid of each petri plate was replaced with bottom part of another petri plate with same size containing PDA medium, duly inoculated with a 6mm mycelial disc of the test pathogen after 3d of incubation. The pairs of each plate were sealed with parafilm (adhesive tape) and incubated at 28±1^{o}C. The PDA medium without *Trichoderma* isolate in the bottom part of petri plate with respective test pathogen on the upper lid of plate served as control. Three replicates were maintained for each treatment. The assembly was opened after 72h and the observations were recorded by measuring colony diameter of the test pathogen (in mm) in each plate and that of the control plates.

Effect of non-volatile metabolites

The effect of culture filtrate of seven isolates of *Trichoderma* sp. on the test pathogens was studied following the method of Dennis and Webster (1971a). Fifty ml. of sterilized potato dextrose broth taken in 250ml Erlenmeyer conical flask was inoculated with a 5mm mycelial disc of the antagonist cut from the edge of four day old culture. Inoculated flasks were incubated at 28±2ºC for 15 days with constant shaking in orbital shaker. The culture filtrate was harvested by passing it through Whatman No. 1 filter paper, and the filtrate was collected in a sterilized conical flask and sterilized by passing through a cellulose membrane Millipore filter after centrifuging it at 6000 rpm for 15 min.

The mycelial disc (6mm) of the test pathogen was inoculated in the center of the petri plate containing 25% of culture filtrate (PDA + culture filtrate) and incubated at 28±1^{o}C, until the respective pathogen fully covered the plate in control. PDA plates inoculated with the test pathogen but not amended with culture filtrate served as control. Three replications were maintained for each treatment. Periodic observations on the radial growth of the test pathogen were recorded (Khan and Sinha, 2007). The percentage inhibition of the mycelial growth of the pathogen by volatile and non-volatile compounds was calculated as per Garcia *et al*. (1994) using the formula:

$$\text{Percentage of inhibition} = \frac{C-T}{C} \times 100$$

C = Radial growth of pathogen (mm) in control,
T = Radial growth of pathogen (mm) in treatment.

Results and discussion

The species of *Trichoderma* isolated from the rhizosphere soils of tomato and chilli crop identified on the basis of morphological characterization and micrometry observations revealed that they belong to seven different species viz., *Trichoderma viride* (Tv), *T. harzianum* (Th), *T. reesei* (Tr), *T. atroviride* (Tatr), *T. koningii* (Tk), *T. pseudokoningii* (Tps) and *T. virens* (Tvrn). *T. viride* was the most predominant species occurring in the present soil samples followed by Th, Tvrn, Tps, Tr, Tk, Tatr. The morphological characterization of these antagonistic isolates was accomplished on the basis of colony color, texture, growth patterns, size of phialides and phialospores.

The test pathogens identified were *Fusarium oxysporum* f. sp. *lycopersici* (Fox), *Alternaria solani* (As), *Aspergillus niger* (An) and *Macrophomina phaseolina* (Mp). The pathogenecity tested pathogens positive were multiplied on PDA slants for storage.

Observations on the growth and colonization of the test pathogens in dual culture screening by the antagonistic isolates (Table 1.) proved that different

species of *Trichoderma* differed in their ability to suppress the growth of the test pathogens. Tps, Tatr and Tvrn showed high antagonism against Fox, As and Mp, Tr against As and Mp, Tk against Fox and Mp, Tv against As by completely overgrowing (100% overgrowth) the colonies of the pathogen and were rated R1 as per Bell's ranking (Table 1.). Tps and Tvrn completely overgrew the colonies of Fox and As, Tr overgrew As in 72 hrs. and it took 96 hrs for Tv to overlap As and Tatr to overlap Fox and As. However, it took 120 hrs by Tk, Tps, Tr, Tatr and Tvrn to completely overgrow Mp and Tk to overgrow Fox. The fast growing isolates caused more inhibition of the pathogen and may be due to mycoparasitism and competition for space and nutrients. Harman *et al*. (1980) had suggested that mycoparasitism was the principle mechanism involved in controlling Pythium damping-off of pea seed. Hyphal parasitism of *Pythium* sp. by *Trichoderma* was also observed *in vitro* by many workers (Lifshitz *et al*., 1986; Kumar and Hooda, 2007).

Tps and Tvrn proved to be superior on account of their faster growth attained against Fox and As (took 24 hrs to contact). Tv was able to overgrow partially over An and Mp, Tk over As, Tatr over An. However, Tv was unable to colonize Fox, Th was unable to overgrow Fox, As and Mp, Tps on An, Tr on Fox and were given Bell's ranking R4. These were locked at the point of contact exhibiting poor hyperparasitic potential against the pathogen. Th, Tk and Tvrn showed no contact even after incubating for 9 days. Slower growth rate and poor competitive ability of these isolates in dual culture is an indication of their poor antagonistic potential.

The variation in hyperparasitic potential of different isolates of *Trichoderma* against soil borne fungal pathogens has been reported (Prasad and Rageswaram, 1999; Sarker and Sharma, 2001; Pan and Bhagat, 2007) and the species of *Trichoderma* were differently selective against different fungi (Dennis and Webster, 1971a, b; Wells *et al*., 1972). This phenomenon may probably be correlated with the differences in levels of hydrolytic enzymes produced by each species or isolates when they attack the mycelium of the pathogens. *Trichoderma* spp. are capable of producing extracellular lytic enzymes that are responsible for their antagonistic activity (Elad *et al*., 1982).

Microscopic observations of mycelial mats from the zone of interaction in the dual culture plates showed that Tps, Tk, Tr, Tatr and Tvrn were interacting with the pathogen by growing towards them (pathogen) and coiled around the hyphae. The mycoparasite was observed to produce small knob like structures. These haustorial knob like structures with penetration pegs, penetrate the host and finally dissolve the protoplasm and shrunk hyphae which may lead to lysis (Weindling, 1932).

No hypahal interaction was observed between Th and any of the pathogens, between Tv and Fox and An, Tk and As, Tr and Fox and Tatr and An. Mycoparasitism includes hyphal interaction and parasitism and is the most vital mechanism of antagonism of the fungal antagonist to give protection to the plants against the pathogen attack. Mycoparasitism as a principle mechanism of biological control is favoured by many scientists (Weindling, 1932; Howell, 1982, 2003). The growth of the mycoparasite towards the pathogen indicates a positive tropism probably chemotropism of the parasite towards the host (Chet, 1987).

Biomass production

Maximum biomass production was recorded with *T. virens,* followed by T. *viride, T. harzianum, T. koningii,* and the lowest biomass was recorded in *T. pseudokoningii* (Table 2, Fig. 1).

Volatile compounds

All the isolates of *Trichoderma* spp. tested for antagonism inhibited the radial growth of the test pathogens *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina* by producing volatile and non-volatile inhibitors. Different isolates of *Trichoderma* varied in production of volatile and non-volatile metabolites as they caused different levels of growth inhibition of the test pathogens.

Tps and Tvrn was observed to be the most efficient among the seven *Trichoderma* sp., in inhibiting growth of the test pathogens by producing volatile inhibitors (Table 3.). Tvrn and Tps showed maximum growth inhibition of As (86.6%) after 3d incubation. Maximum growth inhibition of Fox (81.1%), was recorded after 3d incubation by Tvrn, followed by Tps with growth inhibition of 80.0% in 5 days incubated cultures. Observations indicated that young cultures of *Trichoderma* (antagonistic fungi) produced more volatile compounds resulting in maximum growth inhibition of the pathogen. However, the seven *Trichoderma* species showed less percentage of growth inhibition of Mp by the production of volatile compounds.

Non volatile compounds

The non-volatile compounds in culture filtrates of Tk, Tps and Tvrn considerably inhibited the growth of the test pathogens at 25% concentration (Table 3). Maximum inhibition of As and Fox (86.6 and 82.2%) was recorded in the medium amended with 25% culture filtrate of Tvrn, followed by As (83.3%) and Fox (78.9%) with Tps. Culture filtrate *T. koningii* showed growth inhibition of As by 81.1%. Culture filtrate of all the seven *Trichoderma* species showed less growth inhibition of Mp as compared to other test pathogens. However, culture filtrate of Tr showed minimum growth inhibition of Mp (48.9%).

In the present study Tps, Tvrn and Tk produced volatile and non volatile antibiotics which are inhibitory to the growth of Fox, As, An and Mp (with few exceptions). Other species of *Trichoderma* inhibited the radial growth of the test pathogens but comparatively to a lesser extent.

It is clear from the present study that the antagonistic fungus inhibited the growth of test pathogens by the production of volatile and non volatile compounds indicating the main mechanism involved in biocontrol is antibiosis (Shanmugham and Varma, 1999, Hazarika *et al*., 2000). Antibiosis is antagonism mediated by specific or non specific metabolites of microbial origin, by lytic enzymes, volatile compounds and other toxic substances. Cell free culture filtrate has been used to demonstrate the possible role of antibiosis in bio control. It is also important to mention that *Trichoderma* spp. are known to produce a number of antibiotics such as trichodernin, trichodermol, herzianolide (Kucuk and Kivanc, 2004).

The results in the present study showed that all the isolates of *Trichoderma* spp. highly inhibited the growth of the three pathogens. However, Mp was comparatively less inhibited by producing volatile and non volatile inhibitors. It has been observed earlier that antagonistic fungi are specific in their antagonistic activity against specific fungi (Saleem *et al*., 2000). Antagonism by *Trichoderma* spp. against a range of soil borne plant pathogens has been reported earlier (Pan *et al*., 2001; Papavizas, 1985).

Variability in antagonistic potential of different *Trichoderma* spp. against different pathogens was observed in the present study similar to the earlier reports (Pan and Bhagat, 2007). Strong selectivity of the isolates of *Trichoderma* sp. in their antagonistic potential is essential towards a particular pathogen.

Conclusion

Seven different species of *Trichoderma* exhibited maximum growth inhibition against the test pathogens with variability in the antagonistic potential. Tvrn, Tps, Tatr, Tk, Tv and Th showed high antagonistic potential against *Alternaria solani* and *F. oxysporum.* However, *M. phaseolina* was comparatively less inhibited by all the *Trichoderma* species.

Acknowledgements

Dr. A. Hindumathi is very grateful to the Department of Science & Technology, New Delhi for providing fellowship under Women Scientist Scheme-A (WOS-A) with Grant No. SR/WOS-A/LS-498/2011(G).

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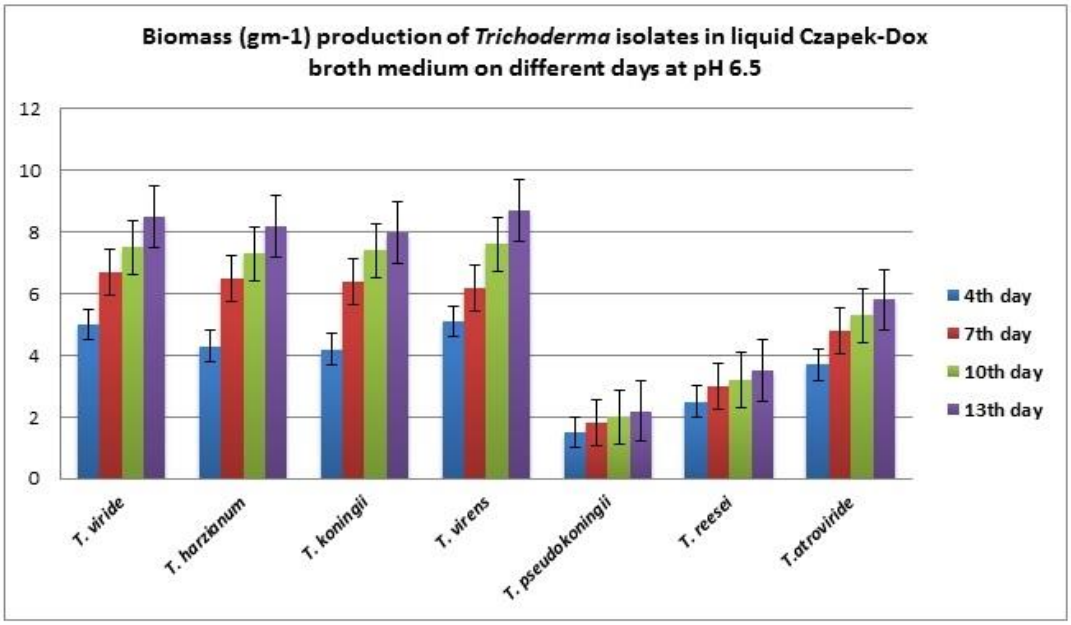

Figure 1. Biomass production of seven *Trichoderma* species in Czapek-Dox broth medium

Figure 2. Effect of volatile compounds produced by *T. viride* on growth of *F. oxysporum*

Table 1. *In vitro* antagonism of seven *Trichoderma* spp. against test pathogens *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina* (BELL'S RANKING)

Trichoderma spp.	Time taken to contact (days)				Time taken to overlap (days)				Bell's Ranking			
	Fox	As	An	Mp	Fox	As	An	Mp	Fox	As	An	Mp
Tv	3	3	4	2	Lkd	4	50%	80%	R4	R1	R3	R2
Th	3	2	NC	2	Lkd	Lkd	NC	Lkd	R4	R4	R4	R4
Tk	3	2	NC	2	5	25%	NC	5	R1	R4	R4	R1
Tps	2	1	2	2	3	3	Lkd	5	R1	R1	R4	R1
Tr	3	2	4	2	Lkd	3	75%	5	R4	R1	R2	R1
Tatr	3	2	4	2	4	4	75%	5	R1	R1	R2	R1
Tvrn	1	1	NC	2	3	3	NC	5	R1	R1	R4	R1

Tv- *T. viride*; Th- *T. harzianum*; Tk- *T. koningii*; Tps- *T. pseudokoningii*; Tr- *T. reesei*; Tatr- *T. atroviride*; Tvrn- *T. virens;* Fox- *Fusarium oxysporum* f sp. *lycopersici*; As- *Alternaria solani*; An- *Aspergillus niger*; Mp- *Macrophomina phaseolina*; NC- No contact; Lkd- Locked; R1- complete over growth; R2- 75% over growth; R3- 50% over growth; R4- locked at the point of contact.

Table 2. Biomass (gm^{-1}) production of *Trichoderma* isolates in liquid Czapek-Dox broth medium on different days at pH 6.5.

S. No	Isolates	4^{th} day	7^{th} day	10^{th} day	13^{th} day
1.	*T. viride*	5.0	6.7	7.5	8.5
2.	*T. harzianum*	4.3	6.5	7.3	8.2
3.	*T. koningii*	4.2	6.4	7.4	8.0
4.	*T. virens*	5.1	6.2	7.6	8.7
5.	*T. pseudokoningii*	1.5	1.8	2.0	2.2
6.	*T. reesei*	2.5	3.0	3.2	3.5
7.	*T. atroviride*	3.7	4.8	5.3	5.8

The data is the average of three replicates

Table 3. Effect of *Trichoderma* volatile and nonvolatile inhibitors on the growth of the test pathogens *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, *Aspergillus niger* and *Macrophomina phaseolina*

Trichoderma spp.	Volatile compounds		Non volatile compounds	
F. oxysporum	Radial growth (mm)	Inhibition (%)	Radial growth (mm)	Inhibition (%)
T. viride	25.0	72.22	21.0	76.6
T. harzianum	19.0	78.9	21.0	76.6
T. koningii	19.0	78.9	22.0	75.5
T. reesei	22.0	75.5	29.0	67.8
T. atroviride	23.0	74.4	19.0	78.9
T. pseudokonongii	18.0	80.0	19.0	78.9
T. virens	17.0	81.1	16.0	82.2
Control	90.0			
Alternaria solani				
T. viride	42.0	53.2	38.0	57.8
T. harzianum	31.0	65.5	32.0	64.4
T. koningii	20.0	77.8	17.0	81.1
T. reesei	30.0	66.6	28.0	68.9
T. atroviride	20.0	77.8	18.0	80.0
T. pseudokonongii	12.0	86.6	15.0	83.3
T. virens	12.0	86.6	12.0	86.6
Control	90.0			
M. phaseolina				
T. viride	43.0	52.2	38.0	57.8
T. harzianum	48.0	46.6	42.0	53.3
T. koningii	42.0	53.3	38.0	57.8
T. reesei	47.0	47.8	46.0	48.9
T. atroviride	49.0	45.5	40.0	55.5
T. pseudokonongii	38.0	57.8	32.0	64.4
T. virens	44.0	51.1	34.0	62.2
Control	90.0			
Aspergillus niger				
T. viride	34.0	62.2	32.0	64.4
T. harzianum	32.0	64.4	29.0	67.8
T. koningii	30.0	66.6	22.0	75.5
T. reesei	32.0	64.4	29.0	67.8
T. atroviride	33.0	63.3	32.0	64.4
T. pseudokonongii	28.0	68.9	22.0	75.5
T. virens	34.0	62.2	29.0	67.8
Control	90.0			

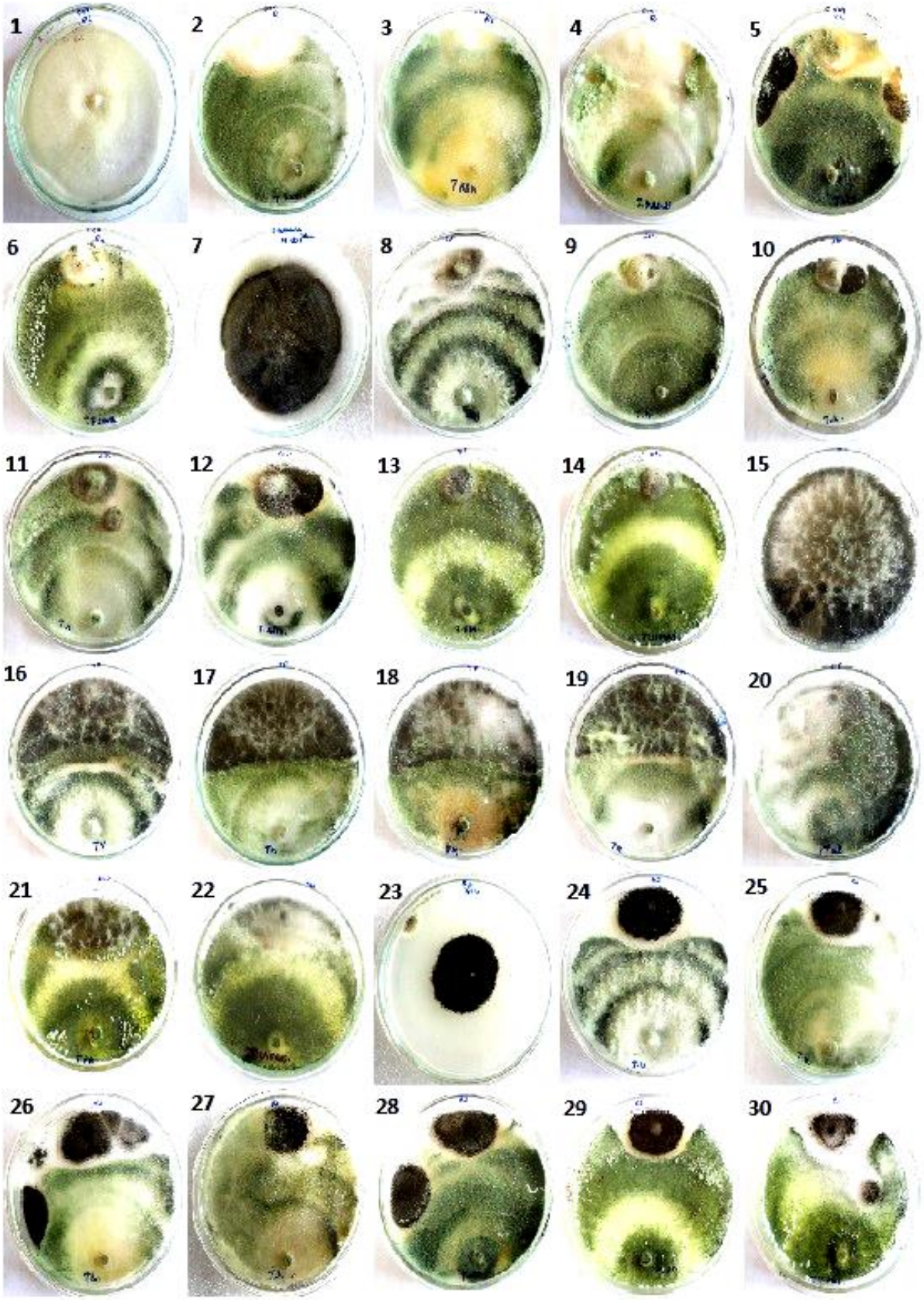

Plate 1. Antagonistic efficacy of seven different *Trichoderma* species against pathogenic fungi. Figs. 1- *Fusarium oxysporum* (control); 2- *F. oxysporum* (Fox)/*T. harzianum* (Th);3- Fox/*T. koningii* (Tk);4- Fox/*T. reseei* (Tr); 5- Fox/*T. atroviride* (Tatr); 6- Fox/*T. pseudokoningii* (Tps); 7- *Alternaria solani* (control); 8- *A. solani* (As)/*T. viride* (Tv); 9- As/Th; 10- As/Tk; 11- As/Tr; 12- As/Tatr; 13- As/Tps; 14- As/*T virens*; (Tvrn); 15- *Macrophomina phaseolina* (control); 16- *M. phaseolina* (Mp)/Tv; 17- Mp/Th; 18- Mp/Tk; 19- Mp/Tr; 20- Mp/Tatr; 21- Mp/Tps; 22- Mp/Tvrn; 23- *Aspergillus niger* (control); 24- *A. niger* (An)/Tv; 25- An/Th; 26- An/Tk; 27- An/Tr; 28- An/Tatr; 29- An/Tps; 30- An/Tvrn