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Screening of different *Trichoderma* species against agriculturally important foliar plant pathogens

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Abstract

Different isolates of *Trichoderma* were isolated from soil samples which were collected from different part of India. These isolates were grouped into four *Trichoderma* species *viz.*, *Trichoderma asperellum* (Ta), *T. harzianum* (Th), *T. pseudokoningii* (Tp) and *T. longibrachiatum* (Tl) based on their morphological characters. Identification of the above isolates was also confirmed through ITS region analysis. These *Trichoderma* isolates were tested for *in vitro* biological control of *Alternaria solani*, *Bipolaris oryzae*, *Pyricularia oryzae* and *Sclerotinia sclerotiorum* which cause serious diseases like early blight (target spot) of tomato and potato, brown leaf spot disease in rice, rice blast disease, and white mold disease in different plants. Under i*n vitro* conditions, all the four species of *Trichoderma* (10 isolates) proved 100% potential inhibition against rice blast pathogen *Pyracularia oryzae*. *T. harzianum* (Th-01) and *T. asperellum* (Ta-10) were effective with 86.6% and 97.7%, growth inhibition of *B. oryzae,* respectively. Among others, *T. pseudokoningii* (Tp-08) and *T. longibrachiatum* (Tl-09) species were particularly efficient in inhibiting growth of *S. sclerotiorum* by 97.8% and 93.3%. *T. longibrachiatum* (Tl-06 and Tl-07) inhibited maximum mycelial growth of *A. solani* by 87.6% and 84.75. However, all the *T. harzianum* isolates showed significantly higher inhibition against *S. sclerotiorum* (CD value 9.430), causing white mold disease. This study led to the selection of potential *Trichoderma* isolates against rice blast, early blight, brown leaf spot in rice and white mold disease in different crops.

Key words

Antagonistic activity, Biocontrol, Dual culture technique, Foliar plant pathogens, *Trichoderma* species

Introduction

A number of plant-associated microbes are free-living and potentially beneficial to plants. Mycoparasitism, a lifestyle where one fungus is parasitic on another fungus, has special relevance when the prey is a plant pathogen, providing a strategy for biological control of pests for plant protection. Probably, the most studied biocontrol agents are species of genus *Trichoderma*. *Trichoderma* species are free-living fungi which are highly interactive in root, soil and foliar environments. The movement toward environment friendly agricultural practices over the past two decades has thus accelerated research in use of fungi capable of biocontrol of plant pathogens (Kubicek *et al.,* 2011). *Trichoderma* spp. play an important role in three-way interaction with plant and pathogen (Lu *et al.,* 2004; Woo *et al.,* 2006). These include production of antibiotics and cell wall degrading enzymes, competition for key nutrients, parasitism, stimulation of plant defense mechanisms and combination of these possibilities.

The internal transcribed spacer (ITS) region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation (Schocha *et al.,* 2012). ITS is also used in some fungi for providing an indication of delimitation by a measure of the genetic distances (Del-Prado *et al.,* 2010). Biocontrol is defined as a combination of different mechanisms working synergistically to achieve disease control (Howell, 2003). *Trichoderma* mycoparasitism combines processes such as nutrient competition (Chet, 1987), secretion of antifungal metabolites (Benítez *et al.,* 2004; Schirmböck *et al.,* 1994; Lorito *et al.,* 1996) and formation of morphological changes such as

coiling around the host and development of appressorium-like structures (Benítez *et al.,* 2004; Lu *et al.,* 2004). *Trichoderma* isolates are known for their ability to control plant pathogens (Elad and Freeman, 2002). Intensive research in biocontrol with *T. harzianum* has been carried out under commercial conditions, and there have been some significant achievements in greenhouse crops and in vineyards (Elad and Shtienberg, 1995). The first biocontrol agent (BCA) to be commercialized, registered and used in greenhouse crops and vineyards was isolate T-39 of *T. harzianum* (TRICHODEX), which effectively controlled *B. cinerea*, *S. sclerotiorum* and *Cladosporium fulvum* diseases in greenhouse-grown tomato and cucumber, and in vineyards (Elad, 1994; 2000 ab). A number of commercial formulations to prevent several diseases in crops as well as in forest trees with economic importance have been developed (Cardoza *et al.,* 2005).

The present investigation evaluated the biological control activity of forty isolates of four different species of *Trichoderma,* originating from different sources against *A. solani, B. oryzae, P. oryzae* and *S. sclerotiorum*, respectively.

Materials and Methods

Collection and isolation of different *Trichoderma* isolates : Soil samples were collected from different parts of India (Table 1). The fungal antagonists were isolated using dilution plate techniques on *Trichoderma* selective medium (TSM) (Elad and Chet, 1983) and purified by single spore method (Rousseau and Halvorso, 1969). The purified and identified cultures of *Trichoderma* spp. were maintained on Potato Dextrose Agar (PDA) medium and stored at 4 °C for further use.

Morphological observations and identification : Morphological observations were recorded from cultures grown on Potato Dextrose Agar at 28 ± 2ºC under ambient laboratory. The microscopic characteristics were observed for more complex conidiophores developing from the characteristic tuft or pustulate areas of conidiation, usually 3 to 5 days after inoculation. Isolates were identified by using identification keys, provided by Rifai (1969) and Bissett (1984, 1991a, 1991b, 1991c). Each ten isolates of *T. asperellum*, *T. harzianum*, *T. longibrachiatum* and *T. pseudokoningii* were included in the present study (Table 1).

DNA extraction, ITS region amplifications and sequencing : DNA was isolated from fresh mycelium using CTAB method (Culling *et al.,* 1992). A region of nuclear rDNA, containing ITS1 and 2, was amplified by PCR using universal primer ITS1 and ITS4 (White *et al.,* 1990), in a thermal cycler (Bioer, Japan), using following parameters: 1 min initial denaturation at 94 °C, followed by 30 cycles of 30 sec denaturation at 94 C°, 1 min primer annealing at 57 °C, 90 sec extension at 72 °C, and a final extension period of 10 min at 72 °C. PCR reaction was electrophoresed on 1.2% agarose minigels (containing 0.5 mgμl^{-1} ethidium bromide) for 1 hr in Tris-acetate buffer (Sambrook *et al.,* 1989). PCR products were revealed under UV light. Direct sequencing of PCR amplicons corresponding to ITS region of ribosomal DNA (rDNA) was performed through outsourcing (Banagalore Genei, Banagalore).

Collection and maintenance of foliar plant pathogens : *Alternaria solani* (4632), *Bipolaris oryzae* (5326), *Pyracularia oryzae* (6810) and *Sclerotinia sclerotiorum* (6094) were procured from Indian Type culture collection (ITCC), IARI, New Delhi. The cultures were maintained on PDA medium and stored at 4 °C for further use.

***In vitro* evaluation of *Trichoderma* spp. against foliar plant pathogens**

Dual culture technique : The dual culture technique described by Dennis and Webster (Dennis and Webster, 1971) was used to test the antagonistic ability of *T. asperellum, T. harzianum, T. longibrachiatum* and *T. pseudokoningii* against *A. solani, B. oryzae, P. oryzae* and *S. sclerotiorum.* The foliar plant pathogens and forty different *Trichoderma* isolates were grown on PDA for a week at 28 ± 2ºC in incubator. Disk of 5mm of the target fungus (*A. solani, B. oryzae, P. oryzae* and *S. sclerotiorum*) was cut from periphery and transferred to petriplate with PDA. *T. asperellum, T. harzianum, T. longibrachiatum* and *T. pseudokoningii* were also transferred aseptically in the same plate. Each plate received two disks, one with *Trichoderma* mycelium and another with any of *A. solani, B. oryzae, P. oryzae* and *S. sclerotiorum*, placed 7cm away from each other. The experiment was conducted in three replications for each antagonist. The plates were incubated at 28 ± 2ºC temperatures and observed after six days for growth of antagonist and test fungus. Index of antagonism as per cent growth inhibition of *A. solani, B. oryzae, P. oryzae* and *S. sclerotiorum* was determined using the formula (Watanabe, 1984).

$$PI = (C - T) \times 100 / C$$

Where, PI is the percent inhibition of mycelial growth; C is the radial growth of pathogen in control plates (cm) and T is the radial growth of pathogen in dual culture (cm). Three replicates were taken for each treatment. Deviation of mean was calculated as standard deviation. The data was statistically analysed.

Results and Discussion

A total of 40 isolates of *Trichoderma*, obtained from different places in India, were studied at the species level by using the morphological characteristics and the analyses of their ITS region nucleotide sequences. All these isolates were identified as *T. asperellum* (samules Lieckfeldt and Nirenberg) (10 isolates), *T. harzianum* Rifai (10 isolates), *T. longibrachiatum* Rifai (10 isolates) and *T. pseudokoningii* Rifai (10 isolates). A single product of approximately 560 to 600 bp was obtained from all the PCR amplifications with primers ITS1 and ITS4 for 40 biocontrol isolates of *Trichoderma* spp. The ITS region of forty isolates, representing

four *Trichoderma* species were previously sequenced (Prameela Devi *et al.,* 2012).The ITS1 and 2 oligonucleotide barcode program TrichOKey (Druzhinina *et al.,* 2005) identified 40 isolates at species level. Morphological grouping and speciation matched with molecular speciation based on the above targeted region. Identification, origin, and NCBI GeneBank accession numbers of all the isolates are given in Table 1.

Cuttack isolate Ta-05 and Varanasi isolate Ta-06 inhibited 100% growth of *P. oryzae.* Navasari isolate Ta -10 inhibited 97.7% growth of *B. oryzae*. Rajendra Nagar isolate showed maximum growth inhibition in *S. sclerotiorum* (91.42%). *T. asperellum* isolated from compost (Ta-01 and Ta-03) showed maximum growth inhibition in *A. solani* by 84.44% and 81.33%. Minimum growth inhibition 57.61% was observed in Varanasi isolate Ta-06 against *S. sclerotiorum* (Fig. 1, Fig. 2A).

Table 1 : Details of isolates of *Trichoderma* species used in present study. Ta–*Trichoderma asperellum*, Th – *Trichoderma harzianum*, Tp – *Trichoderma pseudokoningii*, Tl – *Trichoderma longibrachiatum* (Prameela Devi *et al.,* 2012)

Culture Code	ITCC/ Ident. No	Source/Place of collection	NCBI Gene Bank accession Numbers
Ta-01	4177	Compost / Lucknow	JN104481
Ta-03	5593	Compost / New Delhi	JN104483
Ta-04	6703.07	Soil / Barracpore	JN104484
Ta-05	6706.07	Soil /Cuttack	JN104485
Ta-06	6712.07	Soil / Varanasi	JN104486
Ta-07	7269.08	Soil / Rajendra nagar	JN104487
Ta-08	7297.08	Soil / Guntur	JN104488
Ta-09	7453.09	Soil / Navsari	JN104489
Ta-10	7474.09	Soil / Navsari	JN104490
Ta-11	7475.09	Soil / Navsari	JN104491
Th-01	180	Coconut / Kerela	JN039045
Th-02	184	Coconut / Kerela	JN039046
Th-04	2185	Mushroom compost/Solan	JN039048
Th-05	4532	Soil / Kasargod	-
Th-06	5462	Soil / Plampur	JN039049
Th-07	5595	Compost / New Delhi	JN039050
Th-08	6601.07	Soil / Kasargod	JN039051
Th-09	6197.07	Soil / Kasargod	JN039052
Th-10	7121.08	Compost / New Delhi	JN039053
Th-11	7192.08	Soil / Navasari	JN039054
Tp-01	4799	Onion / Pune	JN039058
Tp-02	5454	Chick pea / New Delhi	JN039059
Tp-03	5460	Chick pea / New Delhi	JN039060
Tp-04	6198	Paddy soil / Assam	JN039061
Tp-05	6725.07	Paddy soil / Assam	JN039062
Tp-06	6729.07	Paddy soil / Assam	JN039063
Tp-07	7185.08	Soil / Shimla	JN039064
Tp-09	7189.08	Soil / Shimla	JN039065
Tp-10	7598.09	Soil / Assam	JN039066
Tp-11	7626.09	Soil / Bilaspur	JN039067
Tl-01	3694	Tomato soil / Ludhiana	JN039072
Tl-02	4282	Soil / Jammu	JN039073
Tl-03	4799	Soil / New Delhi	JN039074
Tl-04	5599	Soil / New Delhi	JN039075
Tl-05	6247	Soil / Tirupathi	JN039076
Tl-06	6755	Soil / Siliguri	JN039077
Tl-07	6756	Soil / Siliguri	JN039078
Tl-08	6760.07	Soil / Siliguri	JN039079
Tl-09	6762.07	Soil / Siliguri	JN039080
Tl-10	7062.08	Sewage / Karnal	JN039081

Three isolates of *T. harzianum* (Th-05, Th-06 and Th-11) showed 100% growth inhibition against *P. oryzae.* Kerela isolate Th-01 and Solan isolate Th-04 showed maximum growth inhibition (86.66% and 86.33%) against *B. oryzae,* where as Solan isolate Th-04 and Kasargode isolate Th-05 inhibited *A. solani* by 82.66% and 84.44%, respectively. Palampur isolate Th-06 was found to be most effective against *S. sclerotiorum* with 8.88%. Out of ten isolate, *T. harzianum* Kerela isolate Th-02 showed least inhibition against *S. sclerotiorum* (Fig. 1, Fig. 2B).

T. longibrachiatum isolates from Tirupathi, Siliguri and Karnal were highly antagonistic to *P. oryzae,* totally overgrowing *P. oryzae* within 5-7 days. Tl-09 and Tl-06 inhibited the growth of *S. sclerotiorum* by 93.3% and 91.90%. Tl-07 and Tl-09 was found to be most effective against *B. oryzae* (85.4% and 80.47%). Among 10 isolates of *T. longibrachiatum,* maximum mycelia growth 87.61% and 84.75% of *A. solani* was observed in l-06 7 Tl-07 (Fig. 1, Fig. 2C,).

Radial growth of *P. oryzae* was highly inhibited by *T. pseudokoningii* isolates Tp-01 and Tp-10 (100%). Chickpea isolate Tp-03 was found to be most effective against *S. sclerotiorum* with 97.8%. Assam isolate Tp-10 found most effective against *B. oryzae* with 83.10%. *T. pseudokoningii* was found least effective against *A. solani* (Assam isolate Tp-06) as compared with other foliar plant pathogens. Assam isolate of *T. pseudokoningii* Tp-04 was highly susceptible to pathogen (49.76%) (Fig. 1, Fig 2D).

T. harzianum showed significant inhibition of *S. sclertiorum* (9.430) where as *T. longibrachiatum* showed less inhibition of *P. oryzae* (1.0976). Out of four different *Trichoderma* species validated against four different pathogen none of them showed less significant value.

Trichoderma species were effective in controlling foliar plant pathogens. ITS1 and 2 sequences are sufficient for distinguishing majority of *Hypocrea*/*Trichoderma* species (Druzhinina *et al.,* 2005), they have only limited power to differentiate at the infraspecific level. In the present study, all identified species are very common and known to be cosmopolitan. Internal transcribed spacer (ITS) regions of nuclear ribosomal DNA have been used in phylogenetic analysis in *Trichoderma* at generic and intra-generic levels. ITS sequences have been recently employed as genetic markers for various species (Prameela Devi *et al.,* 2011). All the isolates of fungal antagonists *viz., T. asperellum, T. harzianum, T. pseudokoningii* and *T. longibrachiatum* inhibited mycelial growth

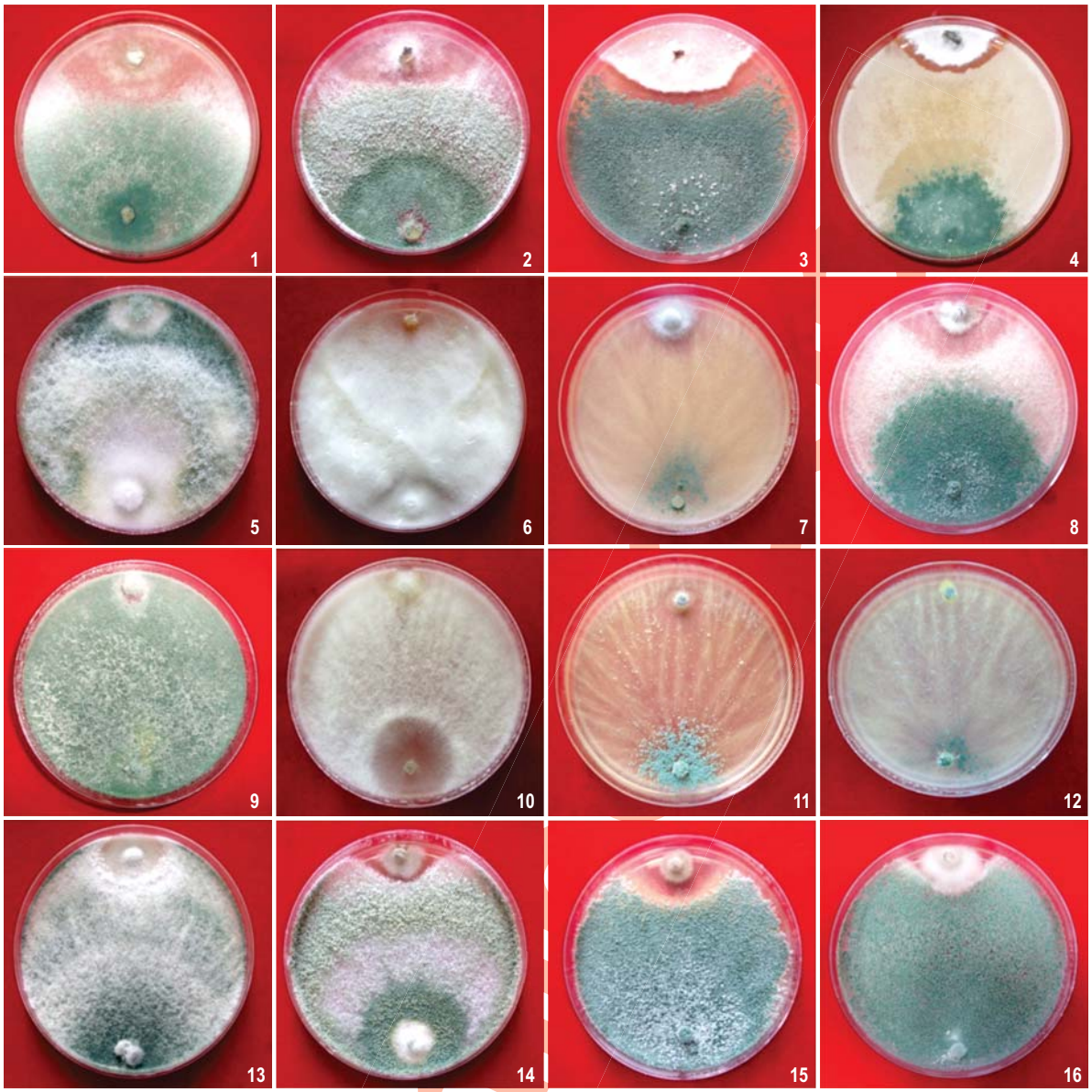

Fig. 1 : 1-4. *Trichoderma* species against *S. Sclerotiorum* (Ta-10, Th-06, Tp-03, Tl-09); **5-8.** *Trichoderma* species against *B. oryzae* (Ta-10, Th-01, Tp-10, Tl-07); **9-12.** *Trichoderma* species against *P. oryzae* (Ta-05, Th-05, Tp-01, Tl-05); **13-16.** *Trichoderma* species against *A. solani* (Ta-03, Th-05, Tp-06, Tl-06).

in four different pathogen. Rice blast is one the most serious fungal diseases in rice growing areas and it is most severe in irrigated rice fields of temperate regions and drought-prone environments. *P. oryzae* is responsible for the most important rice blast disease (blast). *P. oryzae* can cause damage to any aerial part of rice plant, although leaves and panicles (necks) are most commonly affected (Sukanya *et al.,* 2011). All the four species of *Trichoderma* showed 100% growth inhibition of *Pyracularia oryzae*. From each species of *Trichoderma* minimum 2-3 isolates showed maximum growth inhibition against *P. oryzae* (100% growth inhibition). Consolo *et al.* (2012) also investigated the ability of *Trichoderma* isolates to antogonize both foliar and soil-borne fungal plant pathogens, using a dual culture assay against five fungal species: *Alternaria* sp., *Bipolaris sorokiniana*, *Fusarium graminearum*, *F. solani* and *P. oryzae*. Highest inhibition (85%) was observed against *B. sorokiniana* and *P. oryzae* (Consolo *et al.,* 2012). Early blight caused by *Alternaria solani* was found to be the most prevalent leaf disease (Uys *et al.,*

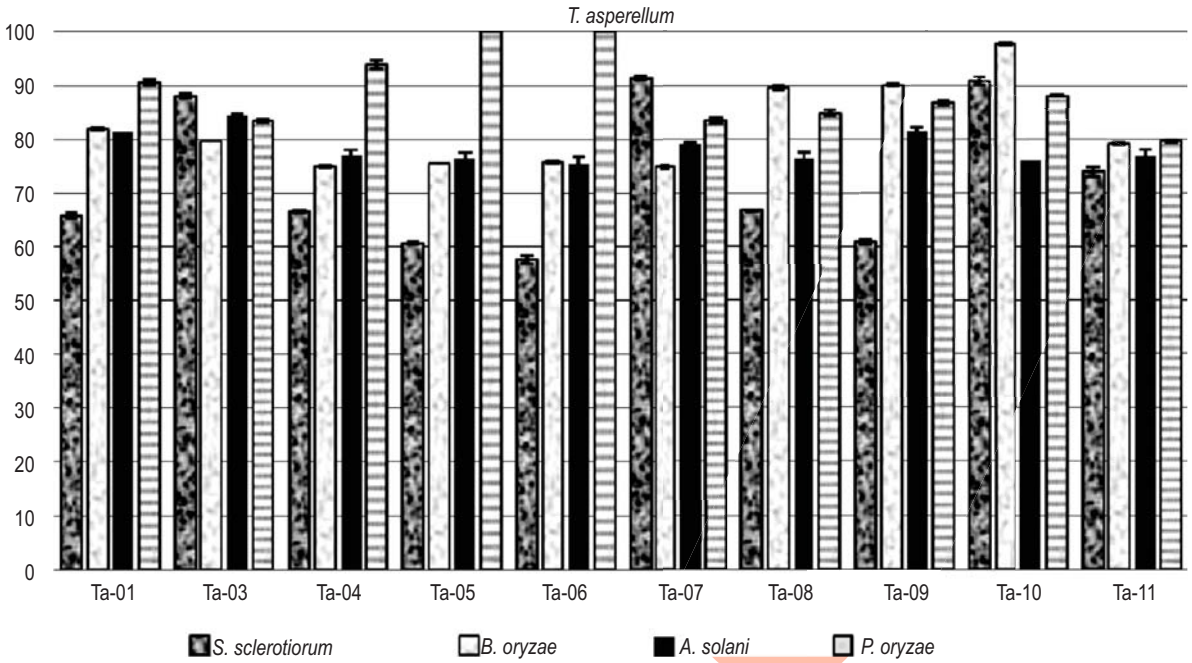

Fig. 2A : Dual-culture assay of *T. asperellum* after 7 days at 25°C with the foliar plant pathogenic fungi *Sclerotinia sclerotiorum*, *Pyracularia oryzae*, *Bipolaris oryzae, Alternaria solani* on PDA

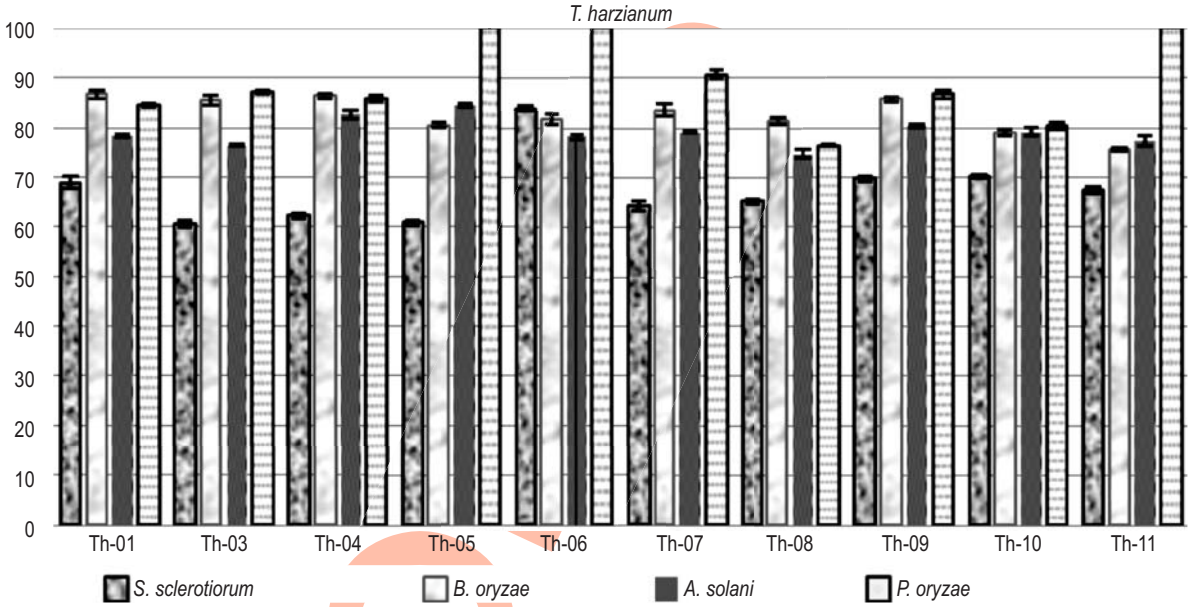

Fig. 2B : Dual-culture assay of *T. harzianum* after 7 days at 25°C with the foliar plant pathogenic fungi *Sclerotinia sclerotiorum*, *Pyracularia oryzae*, *Bipolaris oryzae, Alternaria solani* on PDA

1996). The Siliguri isolate *T. longibrachiatum* (TI – 06) inhibited maximum mycelia growth of *A. solani* by 87%. This result is similar to that of Sanchez *et al.* (2007). *T. longibrachiatum* inhibited the growth of soil borne plant pathogen *Thielaviopsis paradoxa*.

Brown spot, caused by *B. oryzae*, is one of the important rice diseases in the world. Rice brown spot, caused by *B. oryzae,* is a serious disease causing considerable yield loss due to its effect on the quality, number of grains per panicle and kernel weight (Abdel-Fattah *et al.,* 2007). *T. asperellum* (Ta-10) showed

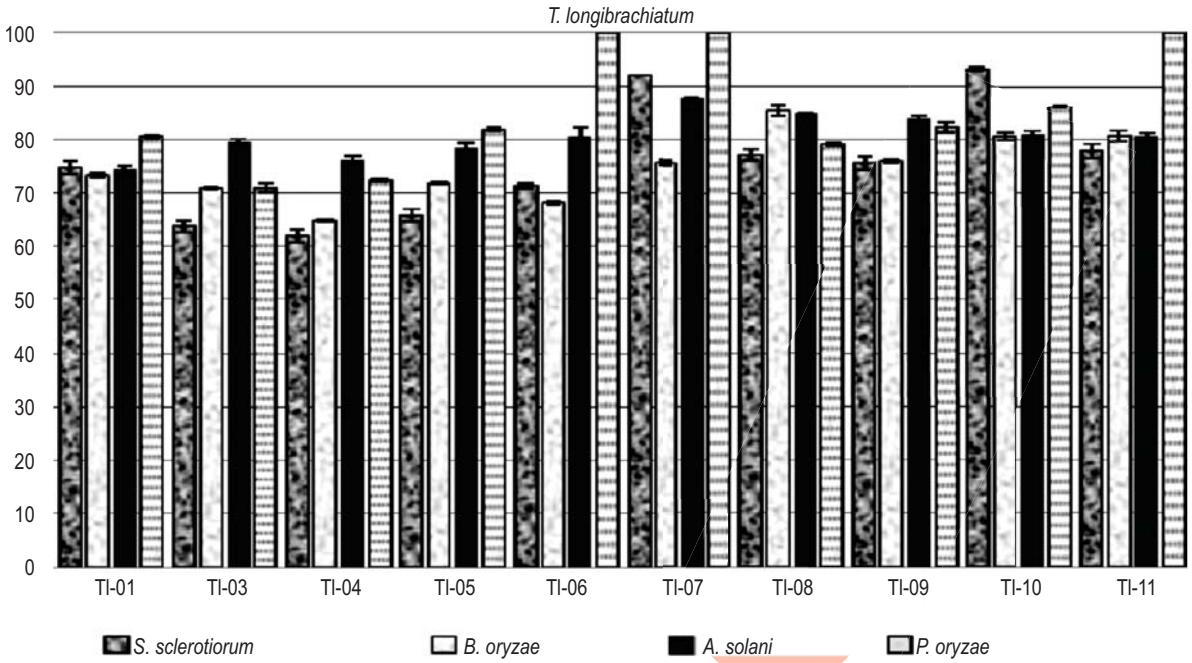

Fig. 2C : Dual-culture assay of *T. longibrachiatum* after 7 days at 25°C with the foliar plant pathogenic fungi *Sclerotinia sclerotiorum*, *Pyracularia oryzae*, *Bipolaris oryzae*, *Alternaria solani* on PDA

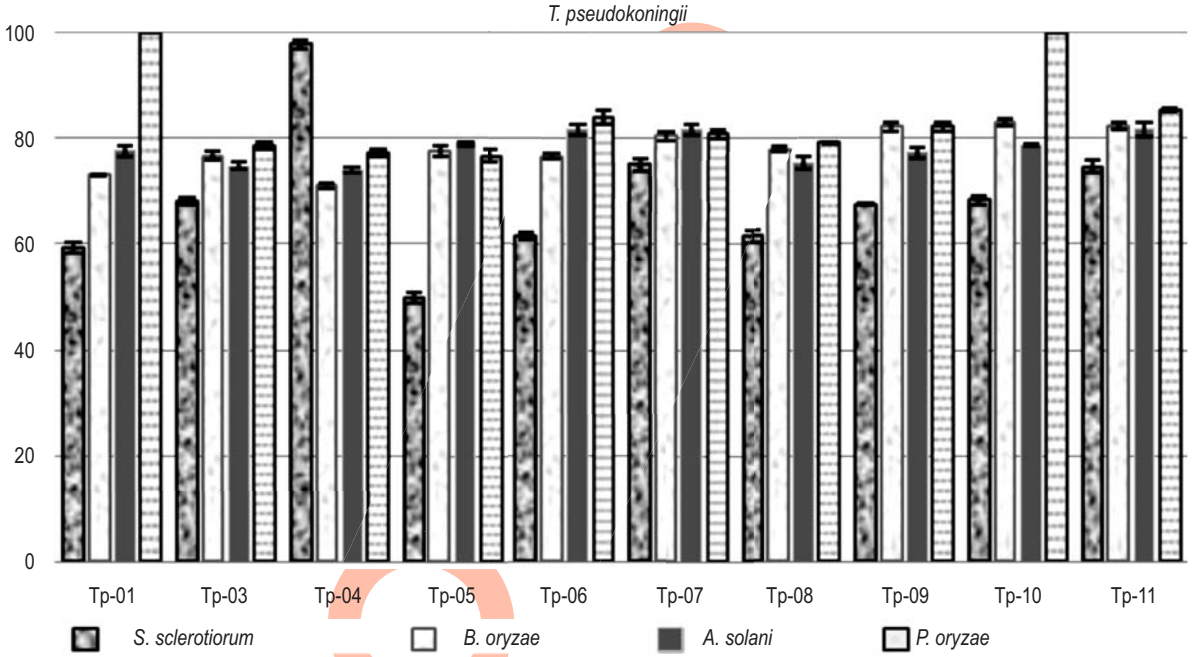

Fig. 2D : Dual-culture assay of *T. pseudokoningii* after 7 days at 25°C with the foliar plant pathogenic fungi *Sclerotinia sclerotiorum*, *Pyracularia oryzae*, *Bipolaris oryzae*, *Alternaria solani* on PDA

highest antagonistic activity against *B. oryzae*. Eleven isolates of *T. asperellum*, tested against *Bipolaris oryzae* and *Fusarium oxysporum* showed technical efficiency exceeding 80% on pathogen (Infante *et al.,* 2011). *Sclerotinia sclerotiorum* attacks a wide range of plants including vegetables like: lettuce, beans, celery, chicory, cucumbers, tomatoes and peas; and many ornamentals, especially plants with hollow stems such as *Dahlia* spp., *Delphinium denudatum*, *Helianthus annuus* and related

yellow daisies (Purdy, 1979). New Delhi isolate *T. pseudokoningii* (Tp-03) showed maximum growth inhibition against (97.8%) *S. sclerotiorum* in *in-vitro* analysis. Which was similar to the reported by Ibarra–Medina *et al.* (2010).

On outdoor, *T. harzanium* had statistically significant effect on white mold (stem rot) disease causing *S. sclerotiorum* in different plants. Overall, *T. harzianum* proved to be a potential bio-control agent against all foliar plant pathogens, particularly against *S. sclerotiorum. T. harzianum* is an effective biocontrol agent for a number of plant fungal diseases (Akhtar *et al.,* 2006 and 2010). Further experimental work is needed to determine the effectiveness of these isolates under different field conditions to consider them as potential bio-control agents as an alternative to chemical pesticides in future.

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