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Biocontrol Efficacy of Different Isolates of *Trichoderma* against Soil Borne Pathogen *Rhizoctonia solani*

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Abstract

In this study, the biocontrol abilities of water-soluble and volatile metabolites of three different isolates of *Trichoderma* (*T. asperellum*, *T. harzianum* and *Trichoderma* spp.) against soil borne plant pathogen *Rhizoctonia solani* were investigated both *in vitro* and *in vivo*. The results showed for the first time that mycelial growth inhibition of the pathogen was 74.4–67.8% with water-soluble metabolites as compared to 15.3–10.6% with volatile metabolites in vitro. *In vivo* antagonistic activity of *Trichoderma* isolates against *R. solani* was evaluated on bean plants under laboratory and greenhouse conditions. We observed that *T. asperellum* was more effective and consistent, lowering disease incidence up to 19.3% in laboratory and 30.5% in green house conditions. These results showed that three isolates of *Trichoderma* could be used as effective biocontrol agents against *R. solani*.

Key words: *Rhizoctonia solani, Trichoderma*, antagonistic activity, biocontrol, soil born pathogen

Introduction

Crop productivity losses due to diseases can result in hunger and starvation especially in developing countries and soil borne fungi are the main causal agents decreasing crop productivity. At present, 1258 different fungal species including *Rhizoctonia solani* have been reported to cause these diseases or are potential threats to crop failure (Ciesielski *et al*., 2009; Consolo *et al*., 2012; Suwannarach *et al*., 2012).

R. solani is a universal fungal pathogen, causal agent of plant roots and lower stem diseases. In Pakistan, *R. solani* occurs as subterranean forms; therefore, chemical control is not a viable choice until the availability of highly selective and efficient fungicides. Control measures of *R. solani* diseases are limited due to wide range of hosts and unavailability of resistant plant varieties (Rouf, 2002). Different strategies to control soil borne pathogens have been hypothesized. Amongst these, biological control has got the attention of most researchers (Benítez *et al*., 2004; Vinale *et al*., 2008; Consolo *et al*., 2012; Chakraborty *et al*., 2013). A large number of soil fungi have been known as potential biological control agents and among them *Trichoderma* exhibits the ability to control the plant pathogens (Punja and Utkhede, 2003; Ting and Choong, 2009; Chaudhary *et al*., 2012). *Trichoderma* are the fast growing filamentous deuteromycetes found in a variety of soils. Due to effective biocontrol abilities of *Trichoderma*; many of its commercial biocontrol products are being marketed in Asia, Europe and USA but none of these are commercially available in developing countries like Pakistan (Consolo *et al*., 2012).

The mechanisms involved in the biocontrol activity of *Trichoderma* spp. against plant pathogens are important in designing effective and safe biocontrol strategies (Wolska *et al*., 2012). Different proposed mechanisms include: mycoparasitism (attack and killing of pathogen) (Anees *et al*., 2010) and competitive inhibition for space and nutrients (Benítez *et al*., 2004). *Trichoderma* are also known to produce different antibiotic substances e.g. gliotoxin, gliovirin, viridin, and trichoviridin (Vinale *et al*., 2008). *Trichoderma* have also been known to inhibit the growth of pathogenic fungi by modifying the rhizosphere (Harman *et al*., 2004). Moreover, infestation of *Trichoderma* in the rhizosphere helps plant to promote nutrient/fertilizer uptake (Yedidia *et al*., 2003), seed germination and photosynthetic rates (Shoresh *et al*., 2010).

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To our knowledge, no work has been carried out so far to explore the biocontrol abilities of indigenous *Trichoderma* populations. The current research was aimed at isolating the indigenous *Trichoderma* spp. and gauging their biological control potential against soil born plant pathogen *R. solani*. Both *in vitro* and *in vivo* trials were carried out to investigate different mechanisms involved in antagonistic activity of *Trichoderma* species. Furthermore, the suppression of disease incidence and related effects on growth were also observed in bean plants.

Experimental

Materials and Methods

Fungal strains. Three *Trichoderma* strains were isolated from agricultural soils as well as obtained from the Fungal Culture Bank of the University of the Punjab Lahore, Pakistan. A highly virulent strain of *R. solani* was isolated from infected bean plants. These fungal strains were maintained at 4°C on Potato Dextrose Agar (PDA) Merck, USA) with periodical sub-culturing on the same medium at 25°C.

Molecular identification of *Trichoderma* strains. The *Trichoderma* isolates were identified according to the protocol of Komoń-Zelazowska *et al.* (2007). DNA isolation was carried out according to Castle *et al.* (1998). The extraction of DNA was done with NucleoMag 96 Plant Kit (Macherey Nagel, Switzerland) and King Fisher technology (Thermo, UK). The primer sequences were; **EF1:** 5'-ATGGGTAAGGA(A/G)GACAAGAC-3' and **EF2:** 5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'. The DNA was quantified by using the Nano drop 1000 (Thermo Scientific, Milan, Italy). For each sample, 1 μl of DNA (50 ng/μl) was amplified and the mixture (20 μl) contained 1 μl of 10× buffer, 0.5 mM of deoxynucleotide triphosphate each, 1 U Taq DNA polymerase (Qiagen, USA), 0.5 mM of each primer and 1.5 mM $MgCl_2$. The PCR program was run as: 95°C, 3 min, 95°C, 1 min; 60°C, 1 min; 72°C, 3 min, 72°C, 5 min for 35 cycles. Five μl of PCR product was run on 1.5% agarose gel containing 1 μl DNA stain SYBR Safe (Invitrogen, USA) in 1×TAE buffer at 3.3 V for 30 minutes and images were obtained with Gel Doc 1000 System (Biorad Lab., USA). Purification of PCR products was done by QIA quick PCR Purification Kit (Qiagen, Milano, Italy) and sequencing was done by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) by BMR Genomics (Padova, Italy).

Homology of the sequences with other deposited nucleotide sequences was checked using basic Blast search program at NCBI and submitted to the website for *Trichoderma* species identification http://www.isth.info/tools/blast/index.php.

Growth profile of *Trichoderma* under different pH and temperature regimes. Five mm mycelial disc was cut from the margins of three days old colonies of each strain by cork borer and placed in the centre of PDA plates and incubated at 20, 25 and 30°C (Chaverri *et al.*, 2003). The growth was optimized at different pH viz. 5, 5.5, 6, 6.5, 7, 7.5. The average colony diameters were measured for 5 days at two dimensions at right angle to each other.

Biocontrol efficacy of *Trichoderma* against *R. solani* in Dual Culture Assay. A 5 mm plug of *Trichoderma* and *R. solani* was cut and incubated at 25°C as described in previous section 2.3. The control plate contained only *R. solani*. The mycelial growth of *Trichoderma* and *R. solani* was recorded after every 24 h, taking the radial growth at right angle to each other and calculating the average (Dennis and Webster, 1971a). Mycoparasitic activity was observed by using light microscope (Axioskop, Germany).

Biocontrol efficacy of water-soluble metabolites of *Trichoderma* against *R. solani*. PDA plates containing cellophane paper were inoculated with 5 mm mycelial discs of 3 days old cultures of *Trichoderma* isolates and incubated at 25°C for 3 days. After 3 days cellophane paper was removed and a 5 mm disc of pathogen was placed on the same PDA plate (Dennis and Webster, 1971b). The control treatment contained only pathogen disc grown without cellophane paper. The cultures were further incubated at 25°C until the colony of pathogen was spread on whole Petri plate in control treatment. The mycelial inhibition of pathogen by *Trichoderma* isolates was calculated using the Eqn. 1 (Edington *et al.*, 1971).

Mycelial Inhibition % = $[(C2 - C1)/C2] \times 100$ (**Eqn. 1**)

Where, C1 = radial mycelial growth of *R. solani* in the presence of *Trichoderma* and
C_2 = radial mycelial growth of *R. solani* in control.

Biocontrol efficacy of volatile metabolites of *Trichoderma* against *R. solani*. The PDA plates were inoculated with 5 mm mycelial discs of 3 days old growing culture of *Trichoderma* isolates. The lid of each plate was replaced with the bottom of other plate inoculated with 5 mm mycelial discs of pathogen. Both plates were sealed together with adhesive tape and incubated at 25°C (Dennis and Webster, 1971c). Control treatment did not contain *Trichoderma* isolate. The mycelial inhibition of pathogen was calculated using Eqn.1.

***In vivo* biocontrol activity of *Trichoderma* species on bean plants.** The bean plants were managed in growth chambers with 12 h photoperiod, 60% humidity and 25°C temperature. The inoculum was prepared according to Pugliese *et al.*, (2008). The *Trichoderma* isolates and pathogen were propagated on sterilized wheat kernel medium (75 g wheat kernel/80 ml H_2O)

and incubated at 25°C in the dark for 10–15 days. *Trichoderma* strains at 5 g/l of inoculum were added to the plastic bags containing steam disinfected peat. Seven days after treatment, the substrate was infested with pathogen at 0.5 g/l and stored at 25°C in growth chambers. The soil of each bag was then transferred to one litre volume pots (10 × 10 × 12 cm) and bean seeds were sown at 5 seeds l^{-1} of peat substrate. The pots were irrigated on a daily basis with sterilized water.

Commercial formulations of *Trichoderma harzianum* ICC 012 2.00% and *Trichoderma viride* ICC 080 2.00% (Remedier®, Isagro Italia Milan, Italy) were used to verify the efficacy of *Trichoderma* isolates. A series of samples treated with a fungicide (Tolclofos-methyl) at the time of sowing of bean seeds were maintained as chemical control. Samples treated only with *R. solani* were used as inoculated (disease) control and non-inoculated (healthy) controls were also maintained throughout the experiment.

Germination was completed five days after sowing and occurrence of any kind of disease was recorded. The plants were uprooted and washed with water. The roots were categorized using scale 0–4 where 0 = healthy plant (no infection), 1 = 25% infected root, 2 = 50% infected root, 3 = 75% infected root, 4 = 100% infected or completely dead plants depending on the appearance of elongate, sunken, red-brown lesions on roots and stems above or below the soil. The Disease index (DI %) was calculated according to Eqn. 2.

$$DI\% = [(n \times 0) + (n \times 0.25) + (n \times 0.5) + (n \times 0.75) + (n \times 1)] / N \times 100 \quad \textbf{(Eqn. 2)}$$

Where, n = number of plants corresponding to each class, N = total number of plants observed. The *Trichoderma* isolates were also assessed for their effect on the growth of bean plants.

Effect of inoculum dose on *in-vivo* biocontrol activity against *R. Solani*. The antagonists and the pathogen were grown on wheat kernel medium as described in previous section. *Trichoderma* isolates were applied at 5 and 1 g/l of peat soil in plastic bags and the bags were kept at green house conditions for one week. After one week, each bag was inoculated with *R. Solani* at 0.25 g/l of soil. The soil of each bag was then transferred to two litres volume pots (12 × 12 × 14 cm) and seeds were sown at 10 seeds/pot. Controls were set up along each treatment.

Experimental layout and Statistical analyses. The experiments were set up in randomized complete block design with four replicates for each treatment. Statistical analysis was carried out using SPSS (version 17.0 ChicagoIL, USA). Analysis of variance (ANOVA) was performed at 5% significance level. Duncan's HSD multiple range test was used as post-hoc analysis to compare means. Pearson's correlation coefficient was calculated to analyze the effect of disease incidence on fresh biomass of bean plants.

Results

Molecular identification of *Trichoderma* species. *Trichoderma* isolates were identified on the basis of 18S RNA gene sequencing with amplification of *tef1* domain at 5' end. The sequences were compared with other nucleotide sequences at NCBI databases using Basic Local Alignment Search Tool (BLAST) and were submitted to Gene Bank (Bankit) for accession numbers. The amplicon of *Trichoderma* TV showed a 99% homology (808/809 and 806/808 bp) with the nucleotide sequence of *T. asperellum* Th021 (AB568376.1) and *T. asperellum* Th016 (AB568375.1) respectively while, *Trichoderma* TK showed 99% homology (738/739 and 738/739 bp) with nucleotide sequence of *T. harzianum* strain CIB T127 (EU279980.1) and *T. harzianum* strain DAOM 167671 (AY605783.1) respectively. The identification was confirmed by searching *tef1* sequences by *Tricho BLAST*.

Growth profile of *Trichoderma* species at different pH and temperature. At 25°C and 30°C, the three fungal species (*T. asperellum, T. harzianum* and *Trichoderma* spp.) showed maximum mycelial growth while, at 20°C the growth rate was considerably reduced and antagonists colonized 1/4th of the medium surface. In acidic pH range *i.e.*, 5–6, the mycelial growth was maximum whilst, moderate growth was observed at pH 6.5 and 7.0 by antagonists. Beyond these pH limits no growth or very little growth (0.9–1.2 cm) was recorded.

Biocontrol efficacy of *Trichoderma* against *R. solani* in dual culture assay. The results demonstrated a strong antagonistic potential of *Trichoderma* against pathogen (Fig. 1). A clear zone of interaction between antagonist and pathogen was observed where the former inhibited the growth of later after making a physical contact. Light microscopic analysis further revealed a typical coiling pattern of *Trichoderma* species around the hyphae of *R. solani* (Fig. 2). This hyphal interaction was initiated after 72 h of incubation. After seven days of incubation, pathogen hypha started to disappear and *T. asperellum*, *T. harzianum* and *Trichoderma* spp. completely overgrew the pathogen.

Biocontrol efficacy of water-soluble and volatile metabolites of *Trichoderma* against *R. solani*. The water-soluble metabolites of all the *Trichoderma* isolates proved to be considerably effective in limiting the growth of *R. solani*. Growth inhibition was significantly higher ($p < 0.01$) with *T. asperellum* (74.4%) followed by *Trichoderma* spp. (70.0%) and *T. harzianum* (67.8%) as compared to control treatment for water-soluble

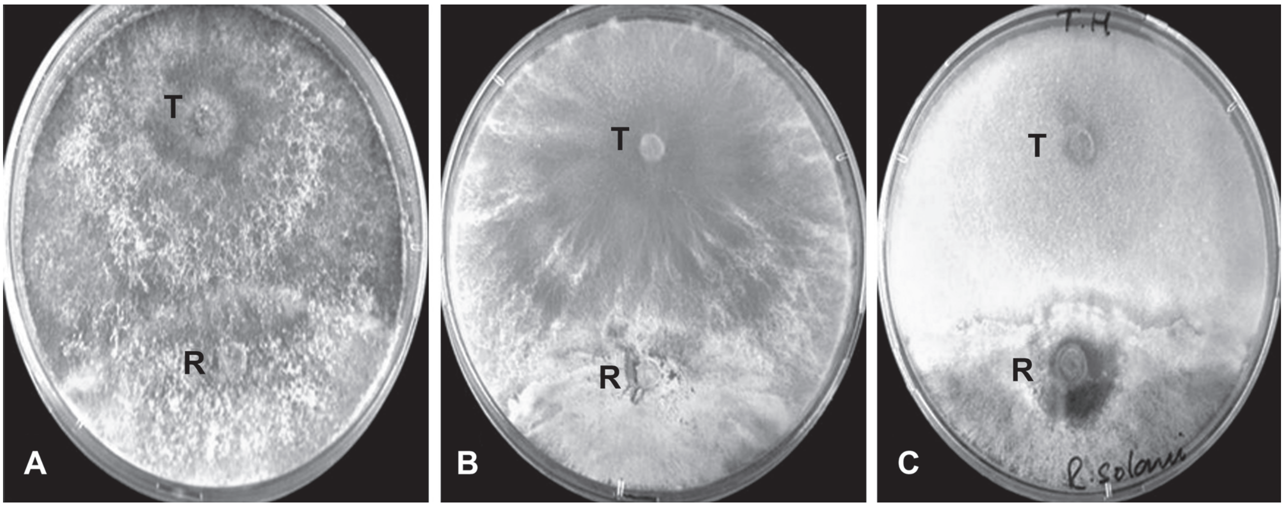

Fig. 1. Antagonistic activity (dual culture assay) of *Trichoderma* isolates (**T**) against *R. solani* (**R**) at 7th day incubated at 25°C.
A: *T. asperellum*; **B:** *T. harzianum*; **C:** *Trichoderma* spp.

metabolites (Table I). All *Trichoderma* isolates exhibited growth inhibition of less than 20% for volatile metabolites (Table I). The values were 15.3% for *T. harzianum*, 11.8% for *T. asperellum* and 10.6% for *Trichoderma* spp. compared to control treatment.

***In vivo* biocontrol activity of *Trichoderma* species against *R. solani*.** The results showed that *T. asperellum* was the most effective biocontrol agent at all application times as it associated with the lowest disease incidence. The relative fresh biomass production was increased compared to inoculated control ($p < 0.01$). When the *Trichoderma* was applied seven days before inoculation of pathogen, all species showed an elevated (19.3 to 26.3%) biocontrol efficacy against *R. solani* compared to inoculated control (54.3%) (Table II). Among the three isolates, *T. asperellum* showed maximum efficacy by lowering the disease incidence up to 19.3% when disease index was 54.3% in inoculated control ($p < 0.01$) (Table II). However, the disease incidence was 23.3% by *T. harzianum* and 26.3% in case of *Trichoderma* spp. Commercial formulation, Remedier®, proved to be less effective ($p < 0.01$) than the tested *Trichoderma* species with a disease incidence of 38.3%. In addition, relative biomass (RW) was decreased as a consequence of pathogen infection (Table II).

All isolates proved to have a positive effect on the growth of bean plants yielding a higher biomass compared to healthy control ones. *T. asperellum* yielded highest relative biomass of 127% ($p < 0.01$) compared to healthy control, followed by *T. harzianum* providing a relative biomass of 113%. *Trichoderma* spp. provided lowest relative biomass (107%) among the species (Table II).

In another experiment, where the antagonist was applied seven days after the pathogen, the best results were observed with *T. asperellum* with 19.7% disease incidence compared to inoculated control (53.3%). This antagonistic behavior was correlated with higher relative biomass *i.e.*, 168% relative weight. Similarly, *T. harzianum* exhibited disease suppression of 23.2%, while the relative biomass was enhanced up to 155%. However, *Trichoderma* spp. showed a disease incidence of 21% but the effect on relative weight of bean plants was low (94%). The commercial formulation, Remedier® showed least biocontrol efficacy (27.7%) and lowest relative biomass (74%) compared to all tested isolates (Table II). When the *Trichoderma* species were evaluated for growth promoting ability, a positive effect on the relative biomass of bean plants was observed. Among the species, *T. harzianum* yielded a highest

Table I
Effect of water-soluble (A) and volatile (B) metabolites of *Trichoderma* on growth inhibition of *R. solani.*

Isolates	Mycelial rowth of *R. solani* (cm)*			% mycelial inhibition at 72 h
	24 h	48 h	24 h	
(A)				
T. asperellum	0.9 ± 0.07	1.0 ± 0.14	1.2 ± 0.07	74.4**
T. harzianum	0.8 ± 0.14	1.2 ± 0.07	1.5 ± 0.07	67.8 b
Trichoderma spp.	1.0 ± 0.07	1.1 ± 0.07	1.4 ± 0.07	70.0 b
Control (*R. Solani*)	1.8 ± 0.14	3.1 ± 0.00	4.5 ± 0.00	0.0 c
(B)				
T. asperellum	1.2 ± 0.00	2.4 ± 0.14	3.8 ± 0.07	11.8 b**
T. harzianum	1.2 ± 0.07	2.5 ± 0.21	3.6 ± 0.00	15.3 a
Trichoderma spp.	1.2 ± 0.00	2.4 ± 0.28	3.8 ± 0.00	10.6 b
Control (*R. Solani*)	1.3 ± 0.14	2.5 ± 0.07	4.3 ± 0.07	0.0 c

* Values are means of four replicates of two independent experiments ± SE.
** Values followed by the different letters in the column are statistically different by Duncan's HSD multiple range Test ($p < 0.05$).

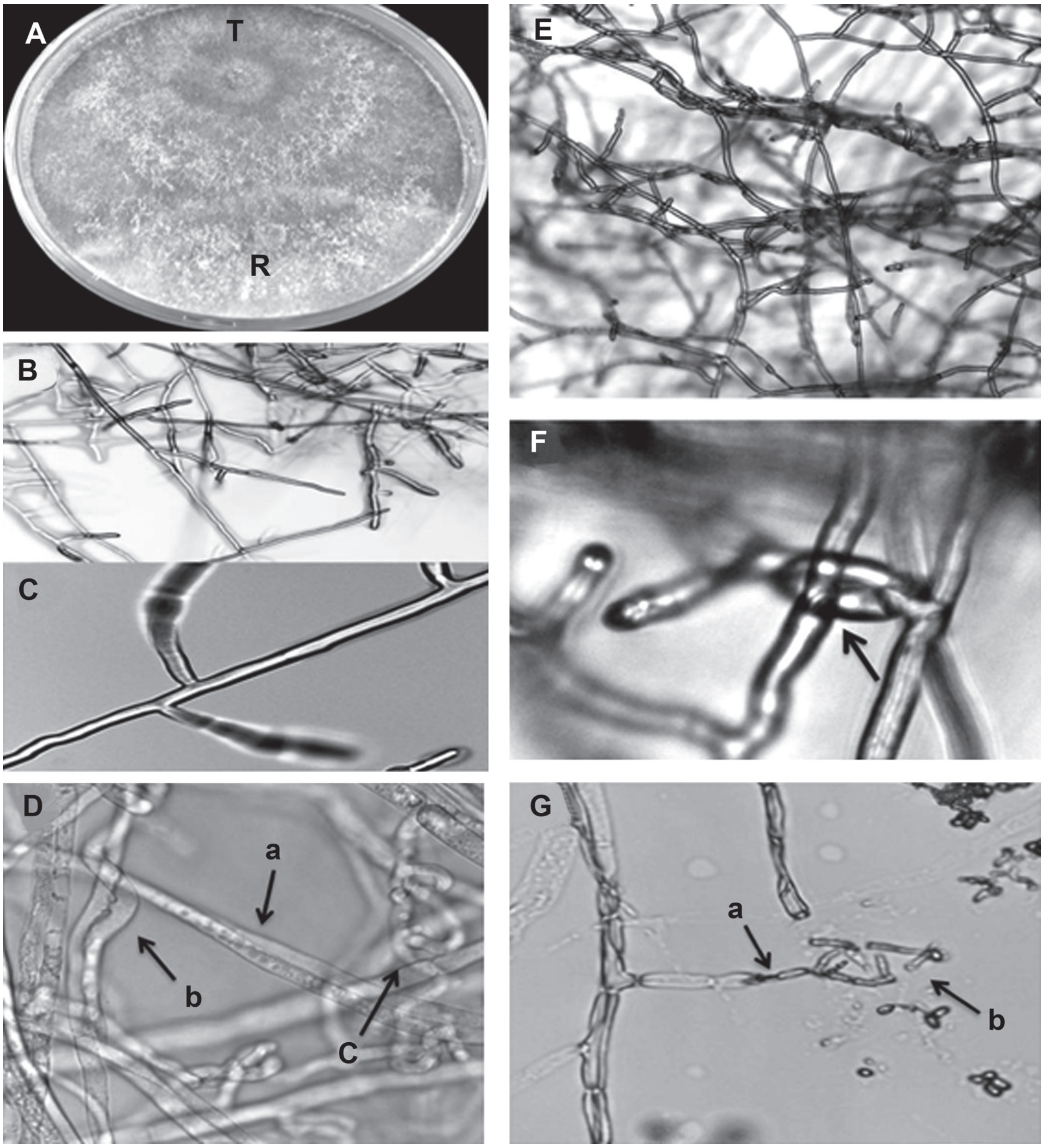

Fig. 2. Hyphal interaction of *T. asperellum* and *R. solani* (light microscope).
A: interaction of *T. asperellum* and *R. solani*; **B:** growing hypha of *T. asperellum*; **C:** hypha of *R. solani* (magnification 150×: bar = 40 μm); **D: a;** lying hypha side by side, **b;** attachment, **c;** coiling of hypha (magnification 150×: bar = 40 μm); **E:** coiling of hypha (magnification 150×: bar = 40 μm); **F:** coiling of hypha at higher magnification (600×: bar = 10 μm); **G:** pathogen hypha after contact, **a;** thicker septa, **b;** fragmented hypha/cell (magnification 150×: bar = 40 μm).

relative biomass of 126% while, *T. asperellum* helped in providing 114% of relative biomass. *Trichoderma* spp. did not show any improvement producing 100% relative biomass (Table II).

Effect of inoculum dose of *Trichoderma s*pecies on *in vivo* biocontrol activity against *R. Solani.* All the *Trichoderma* species showed a higher control efficacy both at high and low dosages, compared to inoculated control. *T. asperellum* was more effective in both trials and showed a decrease in disease incidence with increase in concentration of antagonist, providing a control efficacy of 29.1 and 35.3% ($p < 0.01$), when applied at a dose of 5 and 1 g/l in first trial respectively. However, *Trichoderma* spp. and *T. harzianum* did not show significant differences among the treatments with high and low doses in first trial. In second trial, *T. asperellum* provided the lowest disease incidence but no statistical differences among treatments with dosages ($p < 0.01$), while *Trichoderma* spp. was more effective at a dose of 5 g/l with an efficacy of 35.1% ($p < 0.01$), compared to 39.0% disease incidence with 1 g/l dosage. The *T. harzianum* did not show significant difference in the disease incidence in both trials applied at both dosages. Remedier®, showed a disease index of

Table II
In vivo antagonistic activity of *Trichoderma* species against *R. solani* on bean plants under laboratory conditions (12 h photoperiod, 25°C, 60% relative humidity) expressed as disease incidence % and bean plant fresh biomass.

Isolate	Pathogen (0.5 g/l)	Trichoderma application					
		Seven days before pathogen DI% Plant f. wt. (g) RW			Seven days after pathogen DI% Plant f. wt. (g) RW		
T. harzianum	+	23.3 bc*	23.8 ab	156	23.7 c	23.2 bc	155
T. asperellum	+	19.3 bc	24.0 ab	161	19.7 b	23.7 ab	168
Trichoderma spp.	+	26.3 c	22.0 bc	118	21.0bc	20.7 cd	94
Remedier®	+	38.3 d	17.3 de	19	27.7d	19.9 de	76
T. harzianum	–	0.0 a	23.8 ab	113	0.0 a	26.3 a	126
T. asperellum	–	0.0 a	26.8 a	127	0.0 a	23.9 a	114
Trichoderma spp.	–	0.0 a	22.5 bc	107	0.0 a	20.b cd	100
Remedier®	–	0.0 a	19.4 cde	92	0.0 a	18.8 def	90
Chemical control (Tolclofos methyl)	+	16.7 b	23.2 abc	145	17.7 b	24.4 ab	185
Inoculated control (Only *R. solani*)	+	54.3 e	16.4 e	0	52.0 e	16.8 f	0
Health control (No inoculation)	–	0.0 a	21.1 bcd	100	0.0 a	17.7 ef	100

* Values followed by the letters are statistically different by Duncan's HSD multiple range Test ($p < 0.05$).

Table III
In vivo antagonistic activity of *Trichoderma* species against *R. solani* on bean plants under greenhouse conditions expressed as disease incidence % and fresh biomass.

Isolate	Dosage (g/l)	Pathogen (0.25 g/l)	DI %	Plant f. wt. (g)	RW
T. asperellum	5	+	30.5 bc*	47.6 e	89
T. asperellum	1	+	34.5 cd	42.3 gh	75
Trichoderma spp.	5	+	35.3 cd	47.4 e	81
Trichoderma spp.	1	+	38.5 de	43.3 fgh	62
T. harzianum	5	+	37.1 de	42.8 fgh	59
T. harzianum	1	+	37.3 de	46.7 ef	83
Remedier®	3	+	41.8 e	39.9 h	41
T. asperellum	5	–	0 a	57.4 ab	116
T. asperellum	1	–	0 a	53.2 cd	108
Trichoderma spp.	5	–	0 a	58.5 a	118
Trichoderma spp.	1	–	0 a	54.6 abc	111
T. harzianum	5	–	0 a	52.9 cd	107
T. harzianum	1	–	0 a	54.3 bc	110
Remedier®	3	–	0 a	48.5 e	98
Chemical control (Tolclofos methyl)	–	+	27.2 b	46.1 efg	80
Inoculated control (Only *R. solani*)	–	+	60.4 f	33.2 i	0
Health control (no inoculation)	–	–	0 a	49.4 de	100

* Values followed by letters are statistically different by Duncan's HSD multiple range Test ($p < 0.05$).

48.8 and 34.8% in first and second trial respectively (Table III). Chemical treatment was effective in controlling the disease with an efficacy of 34.4 and 20.0% ($p < 0.01$) in the first and second trial respectively. All the treatments without pathogen showed a high biomass than health control ($p < 0.01$). The *T. asperellum* showed maximum biomass production of 61.2 g in first trial when 5 g/L dose was applied while in second trial the *Trichoderma* spp. yielded a maximum biomass of 60.5 g with same dose. In the treatments with pathogen, the above ground biomass was reduced compared to health control due to consequence of disease (Table III).

The chemical treatment with tolclofos-methyl showed the highest disease suppression and was more effective than the tested fungal strains. The chemical treatment showed 16.7–17.7% disease incidence with

Table IV
Correlation between disease incidence and corresponding biomass of bean plant in different experiments.

Experiment	Pearson's coefficient (r)	Significance
1. *Trichoderma* applied seven days before pathogen (laboratory)	–0.538	p = 0.01
2. *Trichoderma* applied seven days after pathogen (laboratory)	–0.308	p = 0.08
3. Effect of concentration on biocontrol efficacy of *Trichoderma* (greenhouse)	–0.843	p = 0.00

both types of experimental setup. A weak negative correlation was observed for the relative biomass production and disease incidence on bean plants when infested with both pathogen and *Trichoderma* species (r = –0.308). Moreover, the fresh biomass production of bean plants was negatively correlated (r = –0.538) with disease incidence (Table IV).

Discussion

The present study evaluated the biocontrol efficacy of three indigenous strains of *Trichoderma* isolated from agricultural land in Pakistan against soil born plant pathogen *R. solani*.

Two *Trichoderma* isolates out of three used in the present study were identified on the basis of 18S rRNA and they were identified as *T. asperellum* and *T. harzianum*. Both species were previously identified as efficient biocontrol agents against several plant pathogens (Schuster and Schmoll, 2010). Likewise, these isolates showed considerable biocontrol efficacy against *R. solani* in *in vitro* and *in vivo* experimental conditions.

Trichoderma species are distributed worldwide in the rhizospheric regions of plants and around decaying dead biomass (Kubicek *et al.*, 2008). They are extraordinarily able to adjust to the surrounding environmental conditions by regulating their metabolism, growth and reproduction (sporulation). The pH and temperature proved to be major limiting factors affecting the growth profile of *Trichoderma* (Schmoll *et al.*, 2010). The isolates in the present study showed maximum growth at 25°C under acidic pH ranging from 5–6 which establishes their mesophilic nature. These findings are in agreement with Hajieghrari *et al.* (2008); where optimum pH varied from 5–8 and temperature from 25–30°C among different species of *Trichoderma.*

For the control of plant pathogenesis, *Trichoderma* species (mycoparasitism) (Vinale *et al.*, 2006) and/or their extracellular metabolites can be exploited as biocontrol agents or biological fungicides. These metabolites include; volatile and water-soluble metabolites (Eziashi *et al.*, 2006) and secondary metabolites of low molecular weight (Schuster and Schmoll, 2010). The *Trichoderma* isolates studied were not only able to inhibit the growth of pathogen in *in vitro* experiments (Fig. 2, Table I) but also capable of suppressing the disease incidence by the pathogen in *in vivo* trials (Table II, III) confirming their versatile defensive mechanisms. In this context *T. asperellum* proved to be the most effective among the tested isolates. *T. asperellum* has been identified as a potential biocontrol agent in other studies (Osorio-Hernandez *et al.*, 2011) where it showed *in-vitro* inhibition of pathogen in the range of 11–16%. Viterbo *et al.* (2005) characterized a protein kinase TmkA from *T. asperellum,* which had a key role in the regulatory pathways involved in biocontrol activity. *In vitro* studies revealed that *Trichoderma* had comparatively higher growth rates which provide them competitive advantage over the pathogen in availing space and nutrients in the medium. These species also inhibited the growth of the pathogen by secreting certain mycotoxins (Cúndom *et al.*, 2003).

Mycoparasitism is one of the major activities occurring in the antagonist-pathogen interaction, expressed in different steps in a sequence. The detection, attachment, direct penetration, and secretion of fungitoxic enzymes which leads to death of pathogen are major actions in their interface (Harman *et al.*, 2004). The interactions of *Trichoderma* and pathogen in dual culture were observed under light microscope and the hyphal contact between *Trichoderma* and pathogen started after 48–72 h of incubation. Once the contact was established, dense hyphal coiling of *Trichoderma* around *R. solani* hypha, a characteristic response of antagonists was prominent. Similar observations were previously noted against *R. solani* by Almeida *et al.* (2007). The antagonists showed an affinity for the host cell wall which suggests that this may involve chemical bonding between functional sites of carbohydrates present on the cell wall of *Trichoderma* and pathogen which triggers the events leading to host wall penetration (Eziashi *et al.*, 2007).

The production of antifungal compounds also play important role in antagonistic activity of *Trichoderma* species. These include; antibiotics, mycotoxins and low-molecular weight secondary compounds (Schuster and Schmoll, 2010). Our results indicated that all three isolates were able to produce water-soluble metabolites that inhibited the mycelia growth of *R. solani*. *T. asperellum* proved to be the highest producer of these metabolites, while the production of non-volatile metabolites was not obvious. Therefore, the principle mechanism of antagonistic activity against pathogen was speculated as mycoparasitism (Eziashi *et al.*, 2007) and antibiosis or due to the production of secondary metabolites as suggested by Howell (2003). These speculations were supported by the suppression of disease incidence by all *Trichoderma* isolates in *in vivo* trials on bean plants. An increased biocontrol efficacy compared to other isolates and control treatments was provided by *T. asperellum* in terms of application of antagonists before and after the incorporation of pathogen in the soil.

Results in the present study also indicated that the inoculation of antagonist seven days before the pathogen was more effective. These results are in line with De Figueiredo *et al.* (2010) who studied the actions of *Trichoderma* against *Sclerotinia sclerotiorum* in bean plants. The pathogenicity was found to be reduced to 37.04% when antagonist was applied eight days before the pathogen. This approach was also recommended by Lewis and Lumsden (2001).

Interestingly, *Trichoderma* isolates proved to be more effective in controlling the *R. solani* than the commercial formulation Remedier®. This indicates that it is not necessary to apply *Trichoderma* species in complex formulations (Harman, 2000). Additionally, the single strain of *Trichoderma* can be considerably capable of controlling diverse pathogens. Perhaps it would not be possible to commercialize the mixture of biocontrol strains unless there is highly significant success in biological control.

Trichoderma species are well known for their abilities to promote plant growth by colonizing the roots of plants. Their interactions of antagonists with plants enhance the root proliferation and yield production by increasing uptake of nutrients (Harman *et al.*, 2008). The fresh biomass of the bean plants was increased up to 118% when treated with the *Trichoderma* isolates in greenhouse trials as compared to health control (Table III), which significantly proved the ability of *Trichoderma* as a plant growth promoter. These findings are similar to those reported by Pugliese *et al.* (2008) where *Trichoderma* isolates controlled *R. solani* and increased the biomass of bean plants up to 163%. Likewise antagonists prevented 100% mortality of tomato plants coupled with an increase in plant fresh and dry weight (Montealegre *et al.*, 2010). Shaban and El-Bramawy (2011) investigated the biocontrol of damping-off and root rot diseases by combining *Trichoderma* spp. and *Rhizobium* species. They reported an overall improvement in plant growth, seed and fruit production.

In the present study, the different isolates of *Trichoderma* showed as an effective biocontrol agents against *R. solani* though their efficacy varied among isolates and it was highest with *T. asperellum*. The biocontrol efficacy of all *Trichoderma* isolates was even higher than commercial formulation Remedier® in *in vivo* trials. Apart from suppressing the disease profile of bean plants, these isolates showed a considerable effect in promoting their general growth.

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