JEB
Website : www.jeb.co.in
E-mail : editor@jeb.co.in

Journal of Environmental Biology

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Characterization of *Trichoderma* species from vegetable and spice rhizospheres of Andaman Islands for broad spectrum antagonism and plant growth promotion

K. Manigundan[1], K. Sakthivel[1]*, R.K. Gautam[1], Krishna Kumar[2], A. Anantharaj[1], A. Velmurugan[1], P.K. Singh[1] and S. Dam Roy[1]

[1]Division of Field Crop Improvement and Protection, ICAR- Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands-744 101, India

[2]Division of Plant Pathology, ICAR- Indian Agricultural Research Institute, New Delhi-110 012, India

*Corresponding Author E-mail: veluars@gmail.com

Publication Info

Paper received:
31 December 2014

Revised received:
16 June 2015

Accepted:
08 August 2015

Abstract

Trichoderma isolates from different spice and vegetable rhizosphere niche areas of Andaman Islands were characterized for their *in vitro* antagonism, lytic enzyme production and plant growth promotion. The *in vitro* antagonistic results against soil borne pathogens, by dual culture assay revealed that isolates TDK2, TRV 1, TRC 3 showed highest inhibition against *S. oryzae* (63.3%), *F. oxysporum* (40.6%) and *P. aphanidermatum* (40.6%), respectively. In volatile assay the isolate TDK 2 showed highest antagonistic activities against all the three tested pathogens viz., *S. oryzae* (90%), *F. oxysporum* (63.3%) and *P. aphaidermatum* (60%) which was followed by TRV1, TNB6 and TRC3 *Trichoderma* isolates. Lytic enzyme tests revealed that isolates TRC3 and TDK 2 were rich in cellulase (0.485±0.10 U ml^{-1} and 0.702±0.07 U ml^{-1}), chitinase (3.561±0.34 U ml^{-1} and 0.400±0.03 U ml^{-1}) and protease (0.400±0.03 U ml^{-1} and 0.626±0.02 U ml^{-1}) activities respectively. Growth promotion studies, using paper towel method, revealed that four isolates showed good antagonistic and lytic enzyme activities were also found to increase germination percentage, root length and shoot length when treated with tomato and chilli seeds. The molecular characterization of ITS1 region showed prevalence of four different species viz., *T. harzianum, T. asperellum, T. koningiopsis* and *T. aureoviride* among the isolates. The overall results from the above studies revealed that four *Trichoderma* isolates (TDK2, TRV1, TRC3 and TNB6) belonging to *T. aureoviride* and *T. harzianum* from Andaman Islands had better potential for their utilization as broad spectrum bio agents and plant growth promoters either alone or as consortia.

Key words

In vitro antagonism, Plant growth promotion, *Trichoderma*

Introduction

Soil borne filamentous fungal species belonging to genus *Trichoderma* are used as biocontrol agents in a wide range of crop plants for the management of different soil borne pathogens (Haggag and Abd–El Latif 2001; Shali *et al.,* 2010) and also as plant growth promoters (Harman *et al.,* 2004; Monte, 2001; Woo *et al.,* 2006; Lorito *et al.,* 2010; Ainhoa Martínez-Medina *et al.*, 2014). Mycoparasitism has been reported as major antagonistic mechanism of *Tricoderma* where they directly colonize plant pathogens and obtain nutrients from host cell through secretion of various extracellular hydrolytic enzymes (Verma *et al.,* 2007; Savazzini *et al.,* 2009). Cell wall degradation during mycoparasitism is mediated by a set of enzymes including chitinases, b-(1,4)-,b-(1,3)- and b-(1,6)-glucanases and proteases (Gruber and Seidl-Seiboth, 2012). Some species of *Trichoderma* are also involved in plant growth promotion which might be due to production of growth-regulating factors in fungus such as auxin-like secondary metabolites

(Contreras- Cornejo *et al.,* 2009; Chowdappa *et al.,* 2013) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves ACC, the immediate precursor of phytohormone ethylene (Viterbo *et al.,* 2010, Sofo *et al.,* 2012). Soil borne pathogenic fungi play a major role in yield reduction and marketability of many economically important crops worldwide (Rossman, 2009). Successful management of these pathogens has been a serious concern due to their close relationship with soil and surrounding environments. Fungal species like *Macrophomina, Sclerotium, Fusarium* and *Pythium* are widely known to cause root rot, charcoal rot, wilt, damping off symptoms in many crop plants and lead to crop losses even above 50% (El-abbasi *et al.,* 2003). In recent days, use of chemicals for management of these pathogens has been raising serious concern with respect to human and animal health. In addition, many other factors like emergence of fungicide resistant pathogen strains, negative impact of chemicals on soil beneficial microflora and its residual soil toxicity due to improper usage also need attention before formulating suitable control measures for these soil borne pathogens.

It is also pertinent to mention that due to fragile nature and uniqueness of Island ecosystem, various ecofriendly and novel biocontrol agents are highly recommended for control of plant diseases in the Andaman and Nicobar Islands. Hence, use of *Trichoderma* would be best choice for the effective management of soil borne fungal pathogens in the Islands. Therefore, the present study was carried out to identify efficient *Trichoderma* strains having broader antifungal and growth promotion activities through *in vitro* screening, which could be further utilized as potential biocontrol agents in the Islands.

Materials and Methods

Sampling, isolation and maintenance : Rhizosphere soil samples were collected from different spice and vegetable crops of South and North Andaman Islands, India (Fig.1) *Trichoderma* was isolated following the method of Elad (1982). Briefly, serial dilution for each sample was prepared in sterilized water up to the concentration of 10^{-6}, from which 0.5 ml of solution was placed on *Trichoderma* selective media (TSM). All the plates were incubated at 28 ºC for 4 days and *Trichoderma* colonies were purified using hyphal tip technique developed by Dhingra and Sinclair (1985). All the purified colonies were morphologically confirmed and maintained in PDA slants at 4 °C for further studies (Table 1).

Evaluation of antagonistic activity : *Dual culture technique :* Three common soil borne pathogens namely *Sclerotium oryzae* (AN_So3), *Fusarium oxysporum* (AN_Fo2) and *Pythium aphanidermatum* (AN_Pah2) were obtained from culture collections of CIARI, Port Blair were used for dual culture assay. Seven day old-pathogen and

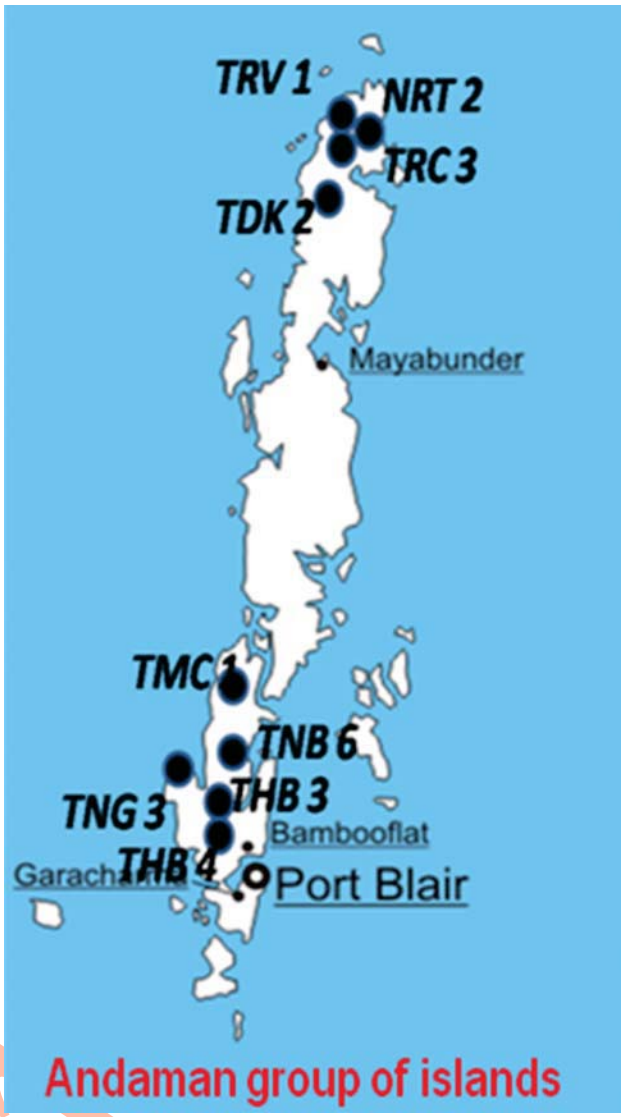

Fig. 1 : Collection sites of nine *Trichoderma* isolates from Andaman Islands, India

Trichoderma isolates grown on Potato Dextrose Agar (PDA) plates were used. Small disks of target fungi and *Trichoderma* strains selected from their actively growing hyphal edge were placed on opposite sides of PDA plates and incubated at room temperature with alternate light and darkness for 7 days. The experiment was replicated thrice and percent growth inhibition was calculated by the formula of I = (C-T)/C x 100, where C is mycelial growth of test organisms in control plate, T is mycelial growth of test organisms in inoculated plate and I is inhibition of mycelial growth.

Evaluation of volatile metabolites : Antagonistic effect of volatile metabolites from *Trichoderma* isolates against three plant pathogens was tested using inverted plate technique (Dennis and Webster, 1971). Briefly, PDA was poured on

both the sides of petriplates and small discs of pathogen and *Trichoderma* isolates were placed at centre of each plate. The plates were then assembled and sealed using paraflim. Control was maintained without *Trichoderma* strain for each test pathogen. All the petridishes were incubated at 28±2˚C for 7 days and observations on radial growth of pathogens were recorded on 7th day of incubation and inhibition percentage was calculated by the formula mentioned above.

Molecular identification of *Trichoderma* : Genomic DNA of *Trichoderma* isolates was extracted using CTAB method as suggested by Raeder and Broda (1985). Isolated DNA was resuspended in 50 µl of TE buffer and checked for its quality using 0.8% agarose gel. For PCR amplification, ITS1, 2 and 5.8S rDNA region of the nuclear rDNA gene cluster was amplified using primers ITS1 and ITS4 (White *et al*., 1990). 50 µl PCR mixture contained 20 ng of template DNA with 2.5 mM concentration of each deoxynucleotide triphosphate, 1 µm concentration of each primer and 3 U of Taq DNA polymerase in 10X Taq buffer A (GeNeI). These reactions were subjected to initial denaturation of 1 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 sec at 55°C and 1.5 min at 72°C with a final extension of 10 min at 72°C using C1000^{TM} thermal cycler (Bio-Rad, Hong Kong). PCR products were resolved using a 1.2% agarose gel and sequencing of purified PCR product was performed at Xcelris, Ahemedabad using ITS1 (forward primer). Sequences were edited and contigs were assembled in DNA baser and compared with GenBank sequences by BLAST analysis. Nucleotide sequence similarities were determined using NCBI database. Phylogenetic dendogram was constructed by the neighbor joining method and tree topologies were evaluated by performing bootstrap analysis of 100 data sets using MEGA 6.1 software.

Assay of hydrolytic enzymes

Preparation of crude extract : Nine isolates of *Trichoderma* were evaluated for their extracellular enzyme activities. For enzyme production, small disks of *Trichoderma* isolates were transferred to 25ml of phosphate buffer (pH 5.5) amended with either 1% carboxymethyl cellulose (CMC), chitin or casein (Kishore *et al.,* 2006) and allowed to grow at 120 rpm for about 7 days. Then the culture supernatants were filtered using Whatman No 2 filter paper and stored at -20˚C as culture supernatant.

Enzyme estimation : Cellulase activity was estimated by the method of Gajera and Vakharia (2012). Mixture of culture supernatant (100 µl) and 100mM sodium citrate buffer (100 µl) pH 5.2 was incubated for 15 min and glucose released was measured by dinitrosalicylic acid method (Sadasivam and Manickam, 1992). Similarly, chitinase activity was measured using N-acetylglucosamine released by dimethylamino benzaldehyde (DMAB) method (Reissig *et al.,* 1955). Protease activity in *Trichoderma* isolates was estimated by measuring free amino acids by ninhydrin method.

Studies on plant growth promotion : Plant growth promoting properties of *Trichoderma* isolates were evaluated *in-vitro* using paper towel method (ISTA, 1993). Briefly, tomato and chilli seeds were surface-sterilized in 1% sodium hypochlorite for 3 min and rinsed four times with sterile water. Seeds were then treated separately with conidial suspension (10^7 conidia ml^{-1}) of each *Trichoderma* strain for 30 min under aseptic condition (Hoyos-Carvajal *et al.,* 2009). Control seeds were treated with sterile water and the experiments were repeated twice with 25 seeds at a time per treatment. After two weeks of incubation at 28±2˚C, shoot and root lengths were measured. Percent germination rates were also calculated.

Statistical analysis : All the experiments were conducted in completely randomized design. Data were subjected to analyses of variance and treatment means were compared by Duncan's multiple range test ($P < 0.01$).

Results and Discussion

The antagonistic ability of *Trichoderma* isolates from diverse vegetable and spices rhizosphere regions of Andaman Islands were evaluated *in-vitro* by dual culture technique and volatile assays (Table 2). Results of dual culture tests revealed seven of the nine *Trichoderma* isolates were found effective in inhibiting the mycelial growth of all the three soil borne fungal pathogens tested. Isolate TDK2 showed highest inhibition (63.4%) against *S. oryzae* when compared to control which was followed by TRV1 (59.7%), TNB6 (58.5%) and TRC3 (54.8%) (Table 2). For another pathogen *F. oxysporum,* TNB6 showed more antagonism (43.3%) which was followed by TRV1 (40%), TMC1 (26.6%) and TRC 3 (26.6%), whereas against *P. aphanidermatum*, isolates TRC3 (40%), TRV1 (37.5%) and TNB6 (37.5%) showed better inhibition of mycelial growth among other *Trichoderma* isolates.

In-vitro evaluation of volatile compounds, using inverted plate assay, revealed that most of the *Trichoderma* species used in the study were rich in their toxic metabolite production against soil borne pathogens. Among the nine *Trichoderma* isolates tested, TDK 2 showed highest antagonistic activities against all the three tested pathogens viz., *S. oryzae* (90%), *F. oxysporum* (63.3%) and *P. aphaidermatum* (60%). For *S. oryzae,* TDK 2 showed maximum mycelial inhibition (90%) followed by TRV 1 (72%) and TNB 6 (68%). Similarly in case of *F. oxysporum,* TRC 3 (36.6%) and TRV 1 (30%) showed best *in-vitro* antagonism next to TDK2 (63.3%.) whereas in *P. aphanidermatum, Trichoderma* isolates TRC3 and TNB 6

Table 1 : Details of *Trichoderma* isolates from vegetable and spice rhizosphere soils of Andaman Islands, India

Isolate name	Crop name	Place of collection	Sporulation	Mycelial colour	ITS based identification (GenBank accession no.)
TRV1	Clove (*Syzygium aromaticum*)	RK Gram	++	White	*T. harzianum* (KJ879441)
TGN3	Nutmeg (*Myristica* sp.)	Guptapara	++	White	*T. harzianum* (KJ879446)
NRT2	Pumpkin (*Cucurbita maxima*)	RK Gram	-	Whitish yellow	*T. asperellum* (KJ879445)
THB4	Bottle gourd (*Lagenaria siceraria*)	Humbrigunj	++	White	*T. koningiopsis* (KJ879444)
THB3	Brinjal (*Solanum melongena*)	Humbrigunj	++	White	*T. koningiopsis* (KJ879443)
TRC3	Cinnamon (*Cinnamomum Zeylanicum*)	RK Gram	+++	Green	*T. harzianum* (KJ879440)
TDK2	Kumda (*Cucurbita maxima*)	DB Gram	-	Whitish yellow	*T. aureoviride* (KJ879447)
TNB6	Brinjal (*Solanum melongena*)	New Mangluton	++	Green	*T. harzianum* (KJ879441)
TMC1	Clove (*Syzygium aromaticum*)	Manjery	+++	Whitish green	*T. harzianum* (KJ879439)

Table 2 : *In vitro* antagonistic potential of *Trichoderma* isolates against *S. oryzae, F. oxysporum* and *P. aphanidermatum*

	Antagonistic activity (%)					
	Isolate name		Dual culture assay		Volatile Assay	
	S. oryzae	***F. oxysporum***	***P. aphanidermatum***	***S. oryzae***	***F. oxysporum***	***P. aphanidermatum***
TRV1	58.4^{b}	40.6^{b}	36.9^{b}	72.0^{b}	30.0^{c}	26.6^{c}
TGN3	49.7^{d}	10.5^{f}	36.9^{b}	30.0^{f}	6.60^{i}	20.0^{e}
NRT2	52.7^{c}	20.0^{e}	-	24.0^{g}	26.60^{d}	20.0^{e}
THB4	42.0^{f}	10.5^{f}	-	40.0^{e}	10.0^{h}	23.3^{d}
THB3	41.1^{f}	20.4^{e}	14.9^{e}	54.0^{d}	16.6^{f}	23.3^{d}
TRC3	54.3^{c}	26.1^{c}	40.6^{a}	68.0^{c}	36.6^{b}	33.3^{b}
TDK2	63.3^{a}	22.7^{d}	14.7^{e}	90.0^{a}	63.3^{a}	60.0^{a}
TNB6	58.0^{b}	42.9^{a}	20.3^{d}	68.0^{c}	23.3^{e}	33.3^{b}
TMC1	46.1^{e}	26.4^{c}	22.2^{c}	18.0^{h}	13.3^{g}	6.6^{f}

*Values are means of three replicates. Values in each column followed by the same letter are not significantly different according to the DMRT method ($P<0.01$)

showed better (33.3%) next to TDK2 (60%) in their volatile activities.

The main mechanisms for biocontrol effects of *Trichoderma* include competition for space and nutrients, mycoparasitism and production of toxic compounds (Savazzini *et al.,* 2009). The overall results from both *in-vitro* antagonist tests indicated that the four isolates viz., TDK2, TRV1, TRC3 and TNB6 showed better antagonism against all the pathogens tested. It indicates that the level of antagonism conferred by each *Trichoderma* isolate varied with different pathogens. Hence, selection of specific isolates depends upon the nature of pathogen for the effective management of soil borne plant diseases. In this context, the results of the present study is in agreement with previous the reports. Dubey *et al.* (2007) reported that antagonistic capacity of *Trichoderma* varied with different species and also with different isolates of same species. Four of the *Trichoderma* isolates (Nz, Kb2, KB3 and Kf1) were found best among 72 isolates tested against *S. rolsfii* (Khattabi *et al.*, 2004), and three species *viz., T. harzianum*, *T. viride* and *T. aureoviride* were found most effective against *R. solani* (Shalini and Kotasthane, 2007).

To confirm species identity, all the nine isolates were subjected to amplification of fragments of rDNA sequences (including 5.8S gene and flanking ITS1 and ITS2 regions) using the universal fungal primers, ITS1 and ITS4. Sequences obtained were edited and contigs were assembled in DNA based software and compared with GenBank sequences by BLAST analysis. Sequence similarities were determined using NCBI database with the already available sequences in NCBI using BLAST. The results revealed the presence of four species among nine isolates viz., *T. harzianum* (5), *T. koningiopsis* (2), *T. aureoviridae* (1), and *T. asperellum* (1). Krishna Kumar *et al.* (2012) reported coexistence of several species of *Trichoderma* (*T. harzianum, T. erinaceum, T. asperellum, T. ovalisporum, T. viride and T. brevicompactum*) in the Bay Island ecosystem. All the sequences were submitted to GenBank and accession numbers were obtained (KJ879439- KJ879447). Phylogenetic analysis using bootstrap analysis with 100 bootstrap replications generated two major clades. Clade I comprised of two species viz., *T. harzianum* and *T. aureoviride* in two subgroups, which were found to be more effective in *in-vitro* antifungal antagonism and plant growth promoting characters in the present study when compared to

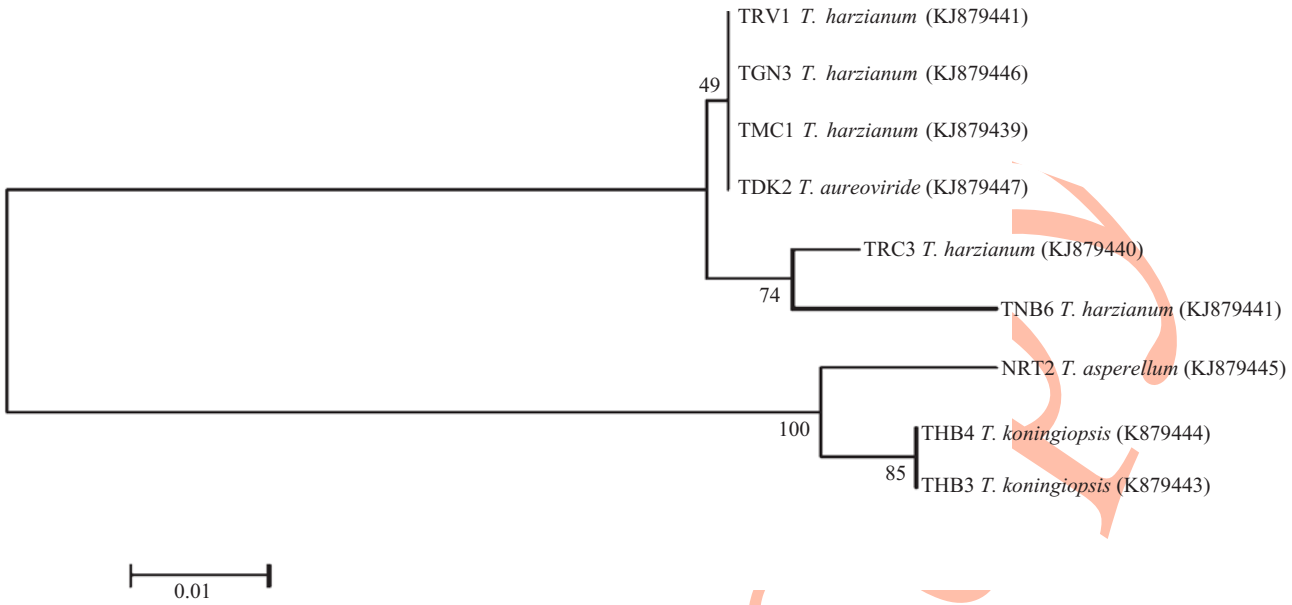

Fig. 2 : Phylogram based on the ITS region of genomic rRNA gene of 9 *Trichoderma* spp. isolated from Andaman Islands

Table 3 : Lytic enzyme activity of *Trichoderma* isolates from Andaman Islands, India

Isolates	Cellulase (U ml^{-1})	Chitinase(U ml^{-1})	Protease (U ml^{-1})
TRV1	0.413±0.09	3.276±0.33	0.111±0.00
TGN3	0.239±0.02	-	0.235±0.01
NRT2	0.393±0.07	-	0.346±0.04
THB4	0.193±0.07	-	0.174±0.02
THB3	0.532±0.02	0.131±0.02	0.410±0.04
TRC3	0.485±0.10	3.561±0.34	0.400±0.03
TDK2	0.702±0.07	2.219±0.28	0.626±0.02
TNB6	0.214±0.07	-	0.222±0.02
TMC1	0.187±0.07	-	0.183±0.02

*Values are mean of three replicates ± SD

other two *Trichoderma* species (*T.asperellum* and *T. koningiopsis)* which were grouped in clade II with two subgroups (Fig. 2).

The most intensively studied lytic enzymes belong to chitinolytic system (chitinases and NAGase) and proteases group (Almeida *et al.,* 2007). Hence, all the *Tichoderma* isolates collected were tested for their *in vitro* lytic enzyme production (Table 3). The results showed that all the isolates could produce cellulase and protease but chitinase activity was observed only in four isolates (TRV1: 3.276±0.33 U ml^{-1}, THB3: 0.131±0.02 U ml^{-1}, TRC3: 3.561±0.34 U ml^{-1} and TDK2: 2.219±0.28 U ml^{-1}). High protease activity was exhibited by TDK2 (0.626±0.02 U ml^{-1})) which was followed by THB3 (0.410±0.04 U ml^{-1}) and TRC3 (0.400±0.03 U ml^{-1}). For cellulase production, maximum enzyme activity was exhibited by TDK2 (0.702±0.07 U ml^{-1}) which was followed by THB3 (0.532±0.02 U ml^{-1}), TRC3 (0.485±0.10 U ml^{-1}) and TRV1 (0.413±0.09 U ml^{-1}). Earlier several studies have also highlighted higher antagonistic activities of *Trichoderma* sp due to production of chitinases (Krishna *et al.,* 2012; Agrawal and Kotasthane, 2012), cellulases (Gajera *et al.,* 2012) and proteases (Kredics, 2005). The results of the present study also confirmed that four among nine isolates expressed high hydrolytic enzyme activities and better antagonistic properties against soil borne fungal pathogens.

All the isolates showed increase in germination percentage as compared to untreated control. Out of the nine isolates TRC3 showed highest germination percentage in both chilli (89%) and tomato (79%). In tomato, TRC 3 and TDK 2 isolates showed highest root length (12.43 cm and 12.30 cm) and shoot length (6.43 cm and 6.03 cm) when compared to other isolates. In case of chilli, TRC3 isolate showed superiority in terms of root length (16.21 cm) and shoot length (3.66 cm) when compared to others. Moncalean *et al.* (2002) reported that enhancement of plant growth by *Trichoderma* sp is mainly due to plant growth regulators,

Table 4 : In vitro growth promotion effect of *Trichoderma* isolates on tomato and chilli crops

	Isolates		Tomato		Chilli	
	Germination %	Shoot length (cm)	Root length (cm)	Germination %	Shoot length(cm)	Root length(cm)
TRV1	68.3^{ab}	6.00^{ab}	9.80^{abc}	71.0^{b}	2.83^{b}	12.68^{e}
TGN3	58.0^{bc}	4.53^{bcd}	7.97^{cd}	50.3^{d}	2.43^{b}	13.38^{de}
NRT2	39.6^{d}	3.57^{d}	8.90^{bcd}	60.0^{c}	3.39^{a}	14.70^{bcd}
THB4	40.3^{d}	5.13^{abcd}	6.93^{d}	71.0^{b}	2.79^{b}	14.86^{abc}
THB3	66.3^{abc}	5.30^{abc}	9.53^{bcd}	70.6^{b}	3.59^{a}	15.09^{ab}
TRC3	79.0^{a}	6.43^{a}	12.43^{a}	89.0^{a}	3.66^{a}	16.21^{a}
TDK2	77.6^{a}	6.03^{abcd}	12.30^{a}	87.6^{a}	2.67^{b}	12.94^{e}
TNB6	51.0^{cd}	5.07^{abcd}	11.40^{ab}	70.3^{b}	2.60^{b}	13.60^{cde}
TMC1	51.0^{cd}	3.67^{cd}	6.93^{d}	59.0^{c}	2.73^{b}	12.42^{e}
Control	41.6^{d}	3.43^{d}	6.73^{d}	49.6^{d}	1.92^{c}	10.28^{f}

Values are means of three replicates. Values in each column followed by the same letter are not significantly different according to the DMRT method ($P < 0.01$)

such as ABA, IAA, GA_3 and so on which act as inducers of plant metabolic processes. Muthukumar *et al.* (2011) reported that chilli seeds treated with culture filtrate of *Trichoderma* strain TVC3 isolated from chilli rhizosphere showed maximum germination percentage, shoot length, root length and vigor index in chilli seedlings, in addition to better mycelial suppression of seedling rot pathogen *P. aphanidermatum* under *in-vitro* conditions. Similar results of plant growth promotion were also observed by earlier workers in tomato and chilli (Fontenelle *et al.,* 2011; Joshi, 2010). Similarly, Hoyos-Carvajal *et al.* (2009) reported that seven isolates of *Trichoderma* significantly improved growth of bean seedlings. Doni *et al.* (2014) also reported that *Trichoderma* sp. SL2 inoculated rice plants exhibited greater plant growth promoting effects like higher net photosynthetic rate, internal CO_2 concentration, water use efficiency, plant height, tiller number, root length and root fresh weight when compared to control and other *Trichoderma* isolates tested.

The results from the above studies revealed that four *Trichoderma* isolates (TDK2, TRV1, TRC3 and TNB6) belonging to *T. aureoviride* and *T. harzianum* from Andaman Islands showed better potential for their utilization as broad spectrum biocontrol agents against soil borne fungal plant pathogens and plant growth promoters, either alone or as consortia. These isolates are being further evaluated in field conditions and disseminated to farmers though various outreach programmes in Andaman and Nicobar Islands.

Acknowledgment

Authors are thankful to the Director, ICAR-CIARI for financial support to carry out the research program.

References

Agrawal, T. and S.A. Kotasthane: Chitinolytic assay of indigenous *Trichoderma* isolates collected from different geographical locations of Chhattisgarh in Central India. *Springer Plus*., **1**, 73 (2012).

Ainhoa Martínez-Medina, Maria Del Mar Alguacil, Jose A. Pascual and Saskia C.M. Van Wees: Phytohormone profiles induced by *Trichoderma* isolates correspond with their biocontrol and plant growth-promoting activity on melon plants. *J. Chem. Ecol*., **40**, 804–815 (2014).

Almeida, F.B., F.M. Cerqueira, R.N. Silva and C.J. Ulhoa: Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani* evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett*., **29**, 1189–1193 (2007).

Chowdappa, P., S.P.M. Kumar, M.J. Lakshmi and K.K. Upreti: Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. *Biol. Cont*., **65**, 109–117 (2013).

Contreras-Cornejo, H.A., L. Macias-Rodríguez, C. Cortes-Penagos and J. Lopez-Bucio: *Trichoderma virens,* a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiol*., **149**, 1579–1592 (2009).

Cruz, L., J.A. Pinter-Toro, T. Benitez and A. Llobell: Purification and characterization of an endo-b-1,3-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J. Bacteriol*., **177**, 1864–1877 (1995).

Cunningham, J.E. and C. Kuiack: Production of citric and oxalic acids and solubilization of calcium-phosphate by *Penicillium*- bilall. *Appl. Environ. Microbial*., **58**, 1451-1458 (1992).

Dennis, C. and J. Webster: Antagonistic properties of species groups of *Trichoderma* II. Production of volatile antibiotics. *Trans. Br. Mycol. Soc*., **57**, 41–48 (1971).

Dhingra, O.D. and J.B. Sinclair: Basics plant pathology methods. CRC Press. Inc. Boca Raton Florida. pp. 13-44 (1985).

Doni, F., I. Anizan, C.M.Z. Che-Radziah and W.Y. Wan Mohtar: Physiological and growth response of rice plants (*Oryza sativa* L.) to *Trichoderma* sp. inoculants. *AMB Express*., **4**, 45 (2014).

Dubey, S., M. Suresh and B. Singh: Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. *Biol. Cont*., **40**, 118–127 (2007).

El-Abbasi, I.H., A.A. El-Wakil and M.M. Satour: Studies on the bioagent *Trichoderma* in Egypt: 1. *In vitro* determination of antagonistic potential of *Trichoderma harzianum* against some plant pathogenic fungi. *Egypt J. Phytopathol*., **31**, 59-74 (2003).

Elad, Y., I. Chet and Y. Henis: Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can. J. Microbiol*., **28**, 719–725 (1982).

Feofila, E.P.: The fungal cell wall: modern concepts of its composition and biological function. *Microbio*., **79**, 723–733 (2010).

Fontenelle, A.D.B., S.D. Guzzo, C.M.M. Lucon and R. Harakava: Growth promotion and induction of resistance in tomato plant against *Xanthomonas euvesicatoria* and *Alternaria solani* by *Trichoderma* spp. *Crop. Protection*., **30**, 1492-1500 (2011).

Gajera, H.P and D.N. Vakharia: Production of Lytic Enzymes by *Trichoderma* Isolates during in vitro Antagonism with *Aspergillus Niger*, the causal agent of collar rot of peanut. *Braz. J. Microbiol*., **43**, 43-52 (2012).

Gruber, S. and V. Seidl-Seiboth: Self versus non-self, fungal cell wall degradation in *Trichoderma*. *Microbio*., **158**, 26–34 (2012).

Haggag, W.M and F.M. Abd-El-Latif: Interaction between vasicular arbuscular mycorrhizae and antagonistic biocontrol micro-organisms on controlling root-rot disease incidence of geranium plants. *J. Biological. Sci*., **1**, 1147-1153 (2001).

Harman, G.E., C.R. Howell, A. Viterbo, I. Chet and M. Lorito: *Trichoderma* species opportunistic, a virulent plant symbionts. *Nat. Rev. Microbiol*., **2**, 43–56 (2004).

Hoyos-Carvajal, L. S. Orduz and J. Bissett: Growth stimulation in bean (*Phaseolus vulgaris* L.) by *Trichoderma*. *Bio. Control*., **51**, 409–416 (2009).

ISTA: International rules for seed testing. Seed Science and Technology 21 (Supplement). pp. 1–288 (1993).

Joshi, B.B., R.P. Bhatt and D. Bahukhandi: Antagonistic and plant growth activity of *Trichoderma* isolates of Western Himalayas. *J. Environ. Biol*., **31**, 921-928 (2010).

Khattabi, N., B. Ezzahiri, L. Louali and A. Oihabi: Antagonistic activity of *Trichoderma* isolates against *Sclerotium rolfsii*: screening of efficient isolates from Morocco soils for biological control. *Phytopathol. Mediterr*., **43**, 332–340 (2004).

Kishore, G.K., S. Pande and A.R. Podile: *Pseudomonas aerouginosa* GSE 18 inhibits the cell wall degrading enzymes of *Aspergillus niger* and activates defence-related enzymes of groundnut in control of collar rot disease. *Aus. Pl. Pathol*., **35**,259-263 (2006).

Kredics, L., Z. Antal, A. Szekeres, L. Hatvani, L. Manczinger, C. Vagvolgyi and E. Nagy: Extracellular proteases of *Trichoderma* species. A review. *Acta Microbiol Immunol Hung*., **52**, 169-184 (2005).

Krishna, K., N. Amaresan, S. Bhagat, K. Madhuri and R.C. Srivastava: Isolation and Characterization of *Trichoderma* spp. for Antagonistic Activity against Root Rot and Foliar Pathogens. *Indian J. Microbiol*., **52**, 137–144 (2012).

Kullnig-Gradinger, C.M., G. Szakacs and C.P. Kubicek: Phylogeny and evolution of the genus *Trichoderma*: a multi gene approach. *Mycol. res*., **106**, 757-767 (2002).

Lee, Y.P. and T. Takahashi: An improved colorimetric determination of amino acids with the use of ninhydrin. *Ann. Biochem*., **14**, 71-73 (1966).

Lorito, M., S.L. Woo, G.E. Harman and E. Monte: Translational research on *Trichoderma*: from omics to the field. *Annu. Rev. Phytopathol*., **48**, 395–417 (2010).

Marco, J.L.D., M.C. Valadares-Inglis and C.R. Felix: Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis perniciosa* the causal agent of witch's broom of cocoa. *Braz. J. Microbiol*., **l34**, 33-38 (2003).

Moncalean, P., A. Rodriguez and B. Fernandez: Plant growth regulators as putative physiological markers of developmental stage in *Prunus persica*. *Plant Growth Regul*., **36**, 27–29 (2002).

Moncalean, P., A. Rodriguez and B. Fernandez: Plant growth regulators as putative physiological markers of developmental stage in *Prunus persica*. *Plant. Growth Regul*., **36,** 27–29 (2002).

Monte, E.: Understanding *Trichoderma*: Between agricultural biotechnology and microbial ecology. *Int. Microbiol*., **4**, 1-4 (2001).

Muthukumar, A., K. Eswaran and Sanjeevkumas: Exploitation of *Trichoderma* species on the growth of *pythium aphanidermatum* in chilli. *Braz. J. Microbiol*., **42**, 1598-1607 (2011).

Raeder, U. and P. Broda. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol*., **1**, 17–20 (1985).

Reissig, J.L., J.L. Strominger and L.F. Lefloir: A modified colorimetric method for the estimation of N-acetyl amino sugars. *J. Biol. Chem*., **217,** 959-966 (1995).

Roger, S.U., F.S. Robert, D.L. Barbara, F. Evelyn, A.M. Mark and Z. Michael: Stress recovery during exposure to natural and urban environments. *J. Environ. Psychol*., **11**, 201-230 (1999).

Rossman, A.Y.: The impact of invasive fungi on agricultural ecosystems in the United States. *Biol. Invasions*., **11,** 97–107 (2009).

Sadasivam, S. and A. Manickam: Biochemical Methods for Agricultural Sciences. Wiley Eastern Ltd., New Delhi. pp. 199-201 (1992).

Savazzini, F., C.M.O. Long and I. Pertot: Impact of the biocontrol agent *Trichderma atroviride* SC1 on soil microbial communities of a vineyard in northern Italy. *Soil. Biol. Biochem*., **41**, 1457-1465 (2009).

Shali, A., S. Ghasemi, G. Ahmadian, G. Ranjbar, A. Dehestani, N. Khalesi, E. Motallebi and M. Vahed: *Bacillus pumilus* SG2 chitinases induced and regulated by chitin, show inhibitory activity against *Fusarium graminearum* and *Bipolaris sorokiniana*. *Phytoparasitica*. **38**, 141-147 (2010).

Shalini, S. and A.S. Kotasthane: Parasitism of *Rhizoctonia solani* by strains of *Trichoderma* sp. *EJEAF Che. ISSN*. 1579- 4377 (2007).

Sofo, A., G. Tataranni, C. Xiloyannis, B. Dichio and A. Scopa: Direct effects of *Trichoderma harzianum* strain T-22 on micro propagated shoots of GiSeLa6 (*Prunus cerasus* x *P. canescens*) rootstock. *Environ. Exp. Bot*., **76**, 33–38 (2012).

Verma, M., S.K. Brar, R.D. Tyagi, V. Sahai, D. Prevost, J.R. Valero and R.Y. Surampalli: Bench-scale fermentation of *Trichoderma viride* on waste water sludge: rheology, lytic enzymes and biocontrol activity. *Enzyme. Microb. Technol*., **41**, 764-771 (2007).

Viterbo, A., U. Landau, S. Kim, L. Chernin and I. Chet: Characterization of ACC deaminase from the biocontrol and plant growth-promoting agent *Trichoderma asperellum* T203. *FEMS Microbiol. Lett*. **305**, 42–48 (2010).

White, T.J., T. Bruns, S. Lee and J. Taylor: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols: a guide to methods and applications (Eds.: MA, Innis, DH, Gelfand, JJ, Sninsky, TJ, White). *Academic Press*, San. Diego. pp. 315-322 (1990).

Woo, S.L., F. Scala, M. Ruocco and M. Loritto: The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathol*., **96**, 1061–1070 (2006).

Yedidia, I., Y. Benhamou and I. Chet: Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbio*., **65**, 1061-1070 (1999).