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Biocontrol Fitness of an Indigenous *Trichoderma viride*, isolate NRCL T-01 against *Fusarium solani* and *Alternaria alternata* causing Diseases in Litchi (*Litchi chinensis*)

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A B S T R A C T

Biological control represents an important approach to manage plant diseases. Blights of leaf, panicle and fruits caused by *Alternaria alternata* and the wilt caused by *Fusarium solani* are important diseases of litchi. Hence, as an alternative to chemical fungicides, biological control by *Trichoderma* spp. to manage pathogens of litchi was explored. The aim of this study was to isolate native *Trichoderma* spp. from litchi orchard ecosystem and to evaluate antagonistic potential and its biological fitness so as to develop a commercial formulation for field application to manage litchi diseases. The results showed that out of nine isolates of *Trichoderma* spp. isolated from litchi rhizosphere soil collected from different litchi orchards located in Muzaffarpur, Bihar the *Trichoderma viride* isolate NRCL T-01 showed highest antagonistic activity in dual culture bioassay against litchi pathogens, *A. alternata* (70.5% inhibition) and *F. solani* (60.9% inhibition). The volatile and non-volatile compounds produced by the isolate could effectively inhibit both the pathogens. It could grow well at temperature between 15 to 45 °C, tolerated pH between 4.0 to 7.0 and high salt stress (0.25-1.50 M NaCl). The talc based formulation of the *T. viride* isolate NRCL T-01 effectively controlled litchi wilt pathogen *F. solani* on challenge inoculation in glasshouse condition as well as naturally affected litchi trees in orchards. Additionally, the isolate showed good plant growth promotion activity acting as a biofertilizer and helping air-layers to establish better in fields. Further study is being conducted to validate the potential as a commercially-viable product under farmers' field conditions.

Introduction

Litchi or Lychee (*Litchi chinensis* Sonn.) [Family Sapindaceae] is a tropical and subtropical fruit tree native to the Guangdong and Fujian provinces of China, and now cultivated in many parts of the world. Litchi is extensively grown in China, India, Thailand, Vietnam and the rest of tropical Southeast Asia, the Indian Subcontinent (Papademetriou and Dent, 2002), and more recently in South Africa, Brazil, the Caribbean, Queensland, California and Florida (Crane *et al.*, 2008). The acreage under litchi cultivation in India was 84,000 ha with a production of 585,000 tonnes during 2013-14 (NHB, 2016). Major litchi producing states in India are Bihar, West Bengal, Assam and Jharkhand. Bihar

contributes 45% of total litchi production and has 40% of the acreage (Kumar *et al.,* 2014). Fortunately, litchi is less affected by diseases than many other fruit trees in India. Among the economically important diseases of litchi in India are anthracnose (*C. gloeosporioides*) and twig blight (*C. gloeosporioides* and *Gloeosporium* sp.) at pre-harvest stage (Kumar *et al.,* 2011, 2014), and fruit rots caused by several pathogens including *Alternaria alternata*, *Aspergillus flavus*, *Cylindrocarpon tonkinense*, *B. theobromae* and *C. gloeosporioides* at post-harvest stage (Awasthi *et al.,* 2005; Kumar *et al.,* 2016a, 2016b). Among the new challenges of diseases, blights of leaf, panicle and fruits caused by *Alternaria alternata* (Kumar *et al.,* 2017) and the wilt caused by *Fusarium solani* (Kumar *et al.,* 2011) are important. To manage pathogens of litchi, particularly *Fusarium solani,* biological control by *Trichoderma* spp. could be an effective alternative.

Trichoderma spp. are fungi that are present in nearly all soils and other diverse habitats. They are well-known biocontrol agents (BCAs) due to their ability to antagonize plant pathogens (Benítez *et al.,* 2004; Harman, 2006), to induce plant defense responses against pathogens, with beneficial effects on plant growth and development (Harman *et al.,* 2004), and also to improve photosynthetic efficiency and respiratory activity, by reprogramming plant gene expression (Shoresh *et al.,* 2010). Many *Trichoderma* spp. antagonize phytopathogenic fungi through mycoparasitism (Lorito *et al.,* 1996a), antibiosis (Ghisalberti and Sivasithamparam, 1991), enzyme production (Markovich and Kononova, 2003), and competition for resources (Sivan and Chet, 1989). In most cases, a single *Trichoderma* spp. isolate simultaneously employs more than one of these antagonistic mechanisms to suppress disease-causing fungi. *Trichoderma* spp. are avirulent symbionts that colonize the outermost epidermal layers of plant roots (Yedidia *et al.,* 2000). During plant - *Trichoderma* interaction, numerous elicitors released by the *Trichoderma* hyphae may induce different types of signals transmitted within the plant e.g. by salicylic acid, jasmonic acid or reactive oxygen species, triggering expression of defence proteins (Nawrocka and Małolepsza, 2013). *Trichoderma* spp. as antagonist has so far not been explored for management of litchi diseases in India. Hence, the aim of this study was to isolate native *Trichoderma* spp. from litchi orchard ecosystem and to evaluate antagonistic potential and its biological fitness so as to develop a commercial formulation for field application to manage litchi diseases.

Materials and Methods

Isolation of pathogens

Isolation of *Fusarium solani* was done from root bits of wilted litchi plants whereas *Alternaria alternata* was isolated from blighted leaves (Kumar *et al.,* 2017). Diseased root bits were washed under running tap water to remove surface soil and other contaminant. Isolations of both the fungi were made by surface-disinfesting small fragments of symptomatic root and leaf tissues in 0.5% NaOCl, double-rinsing in sterile water, and plating onto potato dextrose agar (PDA) amended with 0.05 g L^{-1} streptomycin sulphate. Plates were incubated at 28 ±1°C for 6 days and pure cultures were obtained using the hyphal tip method.

Isolation of *Trichoderma* sp.

Twenty random rhizosphere soil samples were collected from different litchi orchards located in Muzaffarpur, Bihar and stored in plastic bags. The soil samples were air dried and isolation was done following the serial dilution technique on *Trichoderma* selective

medium (Elad *et al.*, 1981). Morphologically distinct colonies were picked on the basis of their morphology (Kubicek and Harman, 1998) and purified on PDA following sub-culturing.

Antagonistic activity of *Trichoderma* isolates

The dual culture technique (Cherif and Benhamou, 1990) was used to test the antagonistic ability of *Trichoderma* sp. against two fungal pathogens of litchi namely *A. alternata* and *F. solani.* A 5 mm mycelia disc of both the fungi (*Trichoderma* sp. and phytopathogenic test fungus) cut from the periphery were placed aseptically on PDA plate about 2.0-2.5 cm away from each other. The plates were incubated at 28±1°C for 7 days and observed periodically. The experiment was conducted under Completely Randomized Design (CRD). The experiment was replicated thrice and the growth of the pathogen in both test and control experiments were recorded. Percent inhibition of radial growth (PIRG) was calculated by the formula: PIRG = (C-T)/ C × 100, where C= radial growth of pathogen in control plate, and T = radial growth of pathogen in dual culture with *Trichoderma* sp. (Kumar *et al.*, 2012). *Trichoderma viride* strain NRCG T-09, isolated from groundnut crop rhizosphere, obtained from Directorate of Groundnut Research, Junagadh, Gujarat was used as reference strain for comparison of antagonistic activity.

Pathogen inhibition through non-volatile inhibitors of *Trichoderma*

The effect of non-volatile metabolites on pathogen was studied following the method of Dennis and Webster (1971) and Jash and Pan (2007). Different antagonists were cultured in 100 mL sterile potato dextrose broth in 250 mL Erlenmeyer flask with intermittent shaking. After 10 days, the culture filtrate was passed through Whatman No. 42 filter paper and the filtrate was collected in sterile Erlenmeyer flasks. The culture filtrate was centrifuged at 3000 rpm for 10 min and sterilized by passing through millipore membrane filter paper (0.4 µm pore size). Different volumes of filtrates were added to the molten PDA medium to obtain final concentrations of 5 and 10% (v/v). The medium was poured into Petri plate and inoculated with mycelial plug of pathogen from 4-day old colonies. The Petri plates were incubated at 28±1 °C for 7 days. Control plates were maintained without culture filtrate. Radial mycelial growth of the pathogen (colony diameter) was measured at right angles to each other and the inhibition percentage calculated.

Pathogen inhibition through volatile metabolites of *Trichoderma*

The effect of volatile metabolites produced by the antagonistic microorganisms on pathogens' mycelial growth was determined following the method described by Dennis and Webster (1971) and Schwarze *et al.*, (2012).

The *Trichoderma viride* was centrally inoculated by placing 5 mm mycelial disc taken from 7-day-old culture onto the PDA plate and incubated at 28±1°C for 4 days. Other PDA plates were inoculated centrally with 5-mm disc of pathogen culture.

Then the top of each *Trichoderma*-inoculated plate was replaced with bottom of the PDA plate inoculated with the pathogen. Two plates were sealed together with paraffin tape and further incubated at 28±1°C. Replicates without *Trichoderma* (having 5 mm of sterile PDA medium disc) were used as the control.

Colonies diameter of the pathogen was measured after 5 and 7 days, and the inhibition of mycelia growth was calculated.

Tolerance to temperature

The ability of NRCL *Trichoderma viride* isolate NRCL T-01 to grow at different temperature was assessed by growing the cultures on PDA plates and incubating them at 15, 20, 25, 30, 35, 40, 45 and 50 °C. *Trichoderma* sp. was inoculated in triplicates at the centre of 90 mm PDA plates by placing 5-mm mycelial discs from the margin of colonies. The plates were incubated at different temperature and the radial growth was measured (in mm) everyday up to 7 days of inoculation.

Tolerance to pH

Different pH used for the study was 4, 5, 6, 7, 8 and 9. One hundred mL PDA media were prepared in triplicates and its pH was adjusted by adding HCl or NaOH before autoclaving. Disc of fungal culture was inoculated on the plates and measurement of the radial mycelia growth and sporulation were recorded.

Tolerance to salt concentration

The effect of NaCl was tested on growth of NRCL *Trichoderma viride* isolate NRCL T-01 cultured on PDA medium. PDA was amended with NaCl at 0.25 M, 0.50 M, 0.75 M, 1.00 M, 1.25 M and 1.50 M concentrations and 5-mm mycelial disc was inoculated in each plate and incubated at 28 °C. The diameter of colonies was measured at 24 hour interval up to 7 days.

Evaluation of *Trichoderma* sp. against litchi wilt under glasshouse and field conditions

For evaluation under glasshouse, rhizosphere soil of one year old potted air-layered litchi plants were inoculated with the pure culture of *F. solani* and talc based formulation of *T. viride* isolate NRCL T-01 (having 2×10^6 cfu/g). Fifty gram talc formulation of *T. viride* was applied on top soil and mixed. *F. solani* was inoculated by adopting toothpick inoculation technique as described by Keeling (1982). Plants were inoculated by inserting toothpick tip overgrown with mycelia of *F. solani*. Five tooth pick per plant was inoculated near the rhizosphere of the plant. *Trichoderma* was applied 3 days prior to inoculation of *F. solani*. Five plants inoculated with *F. solani* only served as control.

For evaluation under field conditions, trees of different age in the orchards, affected presumably by wilt pathogen *F. solani* at various times were chosen. A talc based formulation of *Trichoderma viride* isolate NRCL T-01 (having 2×10^6 cfu/g) was applied @ 100-200 g per tree depending on age (<5 year old @100 g/tree and >5 year old @ 200 g/tree). The days to recovery of trees and population count (cfu) of *Trichoderma* in the rhizosphere soil was monitored. *Trichoderma* was added to the soil along with vermicompost (5 kg for bearing trees and for juvenile trees about 2-4 kg/tree) as a food substrate.

Statistical analysis

The data were analyzed using SAS® 9.2 statistical computing software and subjected to analysis of variance (ANOVA). The least significant differences (LSD) between means were computed at 5% significance level ($P < 0.05$).

Results and Discussion

Isolation and identification of pathogens

Fungi isolated from root bits of wilted litchi plants produced fast growing, white and cottony colony on PDA medium. They slightly curved, thick walled macroconidia having 3-4 septa, and had a slightly blunted apical end. Microconidia were oval to kidney shaped, and formed in false heads on very long monophialides. Chlamydospores were

abundant (Fig. 1). Based on these morpho-cultural characteristics, pathogen was identified as *Fusarium solani* that was also confirmed by a former mycologist, Indian Type Culture Collection, New Delhi.

Similarly, fungi isolated from blighted litchi leaves was identified as *Alternaria alternata* based on morpho-cultural characteristics such as grey to black colonies; branched, brownish, septate mycelia and dark brown, obclavate to obpyriform, catenulate conidia borne on short conidiophores.

Isolation of *Trichoderma* spp. and dual culture bioassay

Nine isolates of *Trichoderma* spp. were obtained from a total of 20 litchi rhizosphere soil samples collected from different litchi orchards located in Muzaffarpur, Bihar. All the isolates showed antifungal antagonistic activity in dual culture bioassay against litchi pathogens, *Alternaria alternata* and *Fusarium solani*. The isolate of *Trichoderma viride* NRCL T-01, isolated from NRCL Farm was found to be the most efficient isolate in controlling both *A. alternata* and *F. solani* under *in-vitro* condition (Fig. 2) as compared to eight other isolates of *Trichoderma* spp. (Table 1). PIRG of colony of pathogens by different isolate of *Trichoderma* spp. varied from 41.0 to 70.5. The maximum inhibition of colony growth of *A. alternata* in dual culture was 70.5% by the isolate NRCL T-01 and the biocontrol agent completely overgrew the pathogen in 8 days. Similarly, the maximum inhibition of colony growth of *F. solani* (60.9%) was by the isolate NRCL T-01 and within 6 days, it completely overgrew the pathogen. Thus, the isolate NRCL T-01 was selected for further assay for biological fitness. The results showed that isolate NRCL T-01 could restrict the growth of phytopathogens of litchi in dual culture which prove its efficacy in management of diseases incited by them. The antagonistic activity of *Trichoderma* depends on multiple synergistic mechanisms (Nallathambi *et al.,* 2009; Howell, 2003). The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition and secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Harman *et al.,* 2004).

Pathogen inhibition through volatile metabolites of *Trichoderma*

The results revealed that in presence of volatile metabolites produced by *T. viride,* radial mycelia growth of the pathogens *viz., A. alternata* and *F. solani* were slower than in control (Fig. 3 and 4). Overall growth rate of *A. alternata* after six days of incubation was 8.8 mm/day in presence of the isolate NRCG T-09 and 6.6 mm/day in the presence of the isolate NRCL T-01 of *T. viride* as compared to 11.7 mm/day in control plates. Similarly, growth rate of *F. solani* after six days of incubation was 14.0 mm/day in presence of the isolate NRCG T-09 and 11.9 mm/day in the presence of isolate NRCL T-01 of *T. viride* as compared to 21.5 mm/day in control plates. The data also showed that after 6 day of incubation, there was 24.86% inhibition of growth of *A. alternata* by the *T. viride* isolate NRCG T-09, whereas it was 43.28% by the isolate NRCL T-01. Likewise, after 4 days of incubation, an inhibition of 20.93% in growth of *F. solani* by the isolate NRCG T-09 and 40.69% by the isolate NRCL T-01 was apparent. Thus, results clearly showed the superiority of the local strain NRCL T-01 over NRCG T-09 in controlling both the pathogens of litchi. There is large variety of volatile secondary metabolites produced by *Trichoderma* such as ethylene, hydrogen cyanide, aldehydes and ketones which play an important role in controlling the plant pathogens (Vey *et al.,* 2001).

Fig.1 Asexual spores (microconidia and macroconidia) of *Fusarium solani*

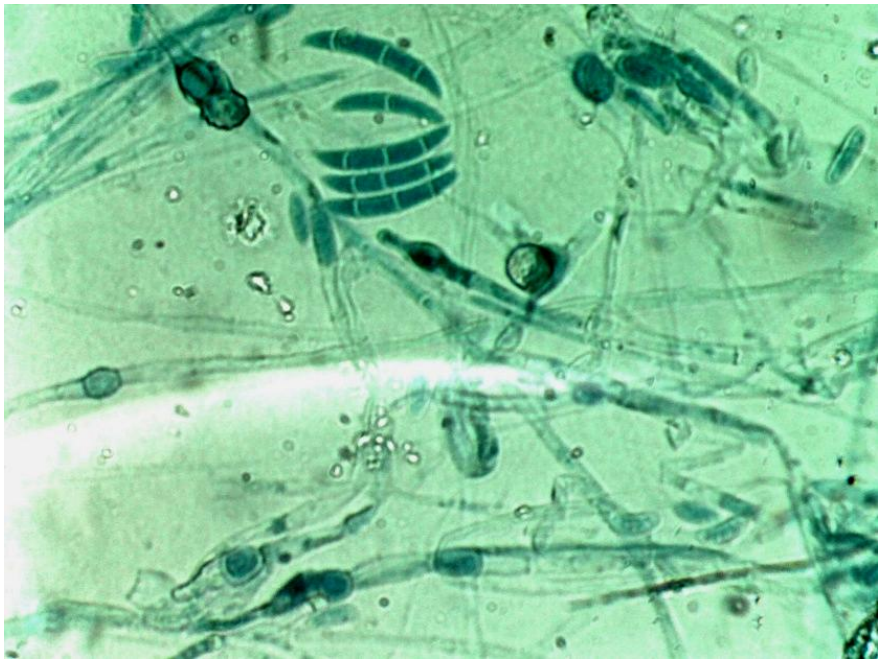

Fig.2 *In vitro* antifungal activity of the *Trichoderma viride* strain NRCL T-01 against *A. alternata* (A, B) and *F. solani* (C, D) on PDA plates

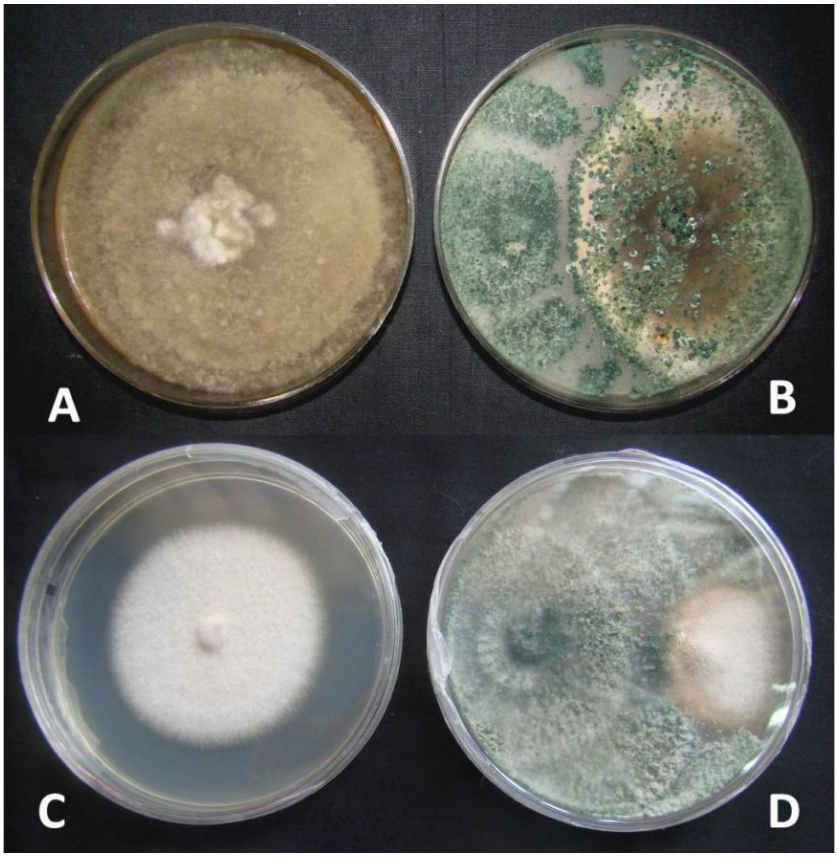

Fig.3 Radial mycelia growth of *Alternaria alternata* in the presence of volatile metabolites emitted by *Trichoderma vride.* The vertical bar indicates standard error (SE) of the means

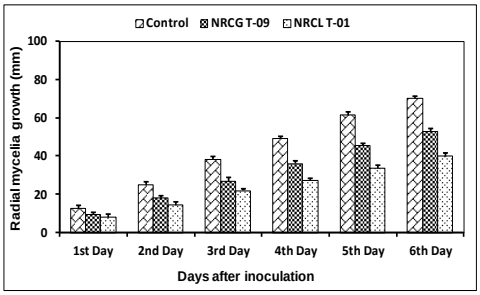

Fig.4 Radial mycelia growth of *Fusarium solani* in the presence of volatile metabolites emitted by *Trichoderma vride.* The vertical bar indicates standard error (SE) of the mean

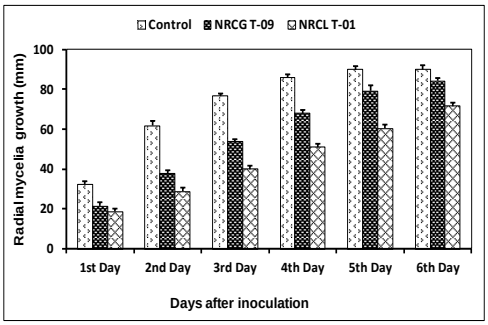

Fig.5 Effect of non-volatile compounds produced by *Trichoderma viride* isolate NRCL T-01 on growth of *Alternaria alternata*

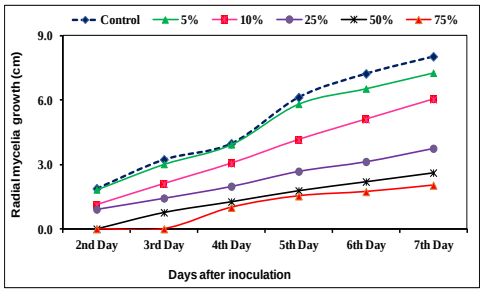

Fig.6 Effect of non-volatile compounds produced by *Trichoderma viride* isolate NRCL T-01 on growth of *Fusarium solani*

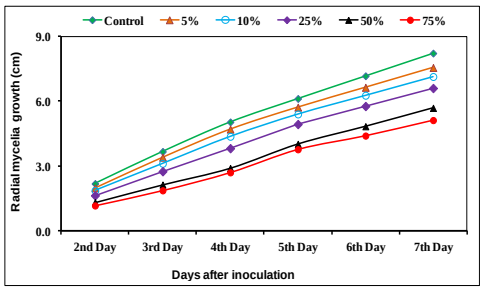

Fig.7 Effect of non-volatile compounds produced by *Trichoderma viride* isolate NRCG T-09 on growth of *Alternaria alternata*

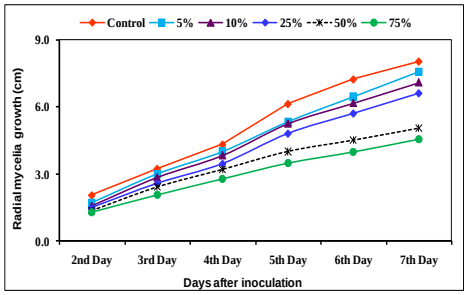

Fig.8 Effect of non-volatile compounds produced by *Trichoderma viride* isolate NRCG T-09 on growth of *Fusarium solani*

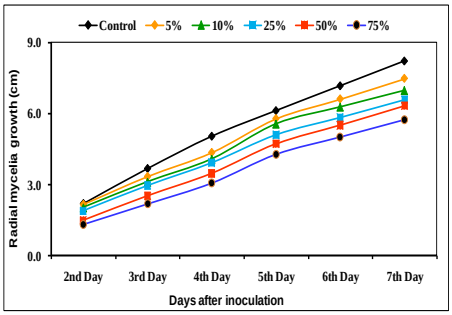

Fig.9 Growth of *Trichoderma viride* isolate NRCL T-01 at different temperatures

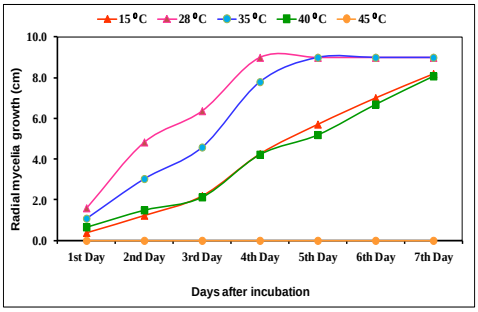

Fig.10 Radial mycelia growth of *Trichoderma viride* isolate NRCL T-01 at different NaCl salt concentration

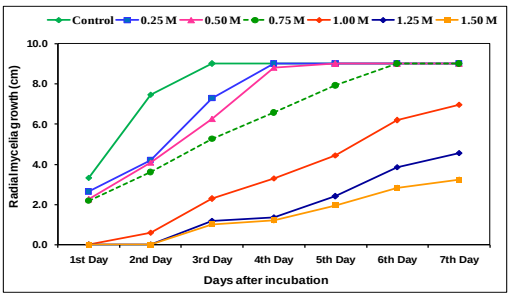

Fig.11 Growth and colony morphology of *Trichoderma viride* isolate NRCL T-01 at different NaCl salt concentration (Left to right: Top row- Control, 0.25 M, 0.50 M, 0.75 M; Bottom row-1.0 M, 1.25 M, 1.50 M)

Fig.12 A litchi tree showing symptoms of wilt (Left) and condition of the tree following recovery due to application of *Trichoderma* (*T. viride* isolate NRCL T-01) at the NRCL experimental farm (Right)

Table.1 Antagonistic activity of isolates of *Trichoderma* spp. against *Alternaria alternata* and *Fusarium solani* in dual culture bioassay

S. No.	Isolate No.	*Trichoderma* species	*Alternaria alternata*			*Fusarium solani*		
			Radial growth in dual culture* (T)	PIRG	Time taken to completely overgrow the pathogen	Radial growth in dual culture* (T)	PIRG	Time taken to completely overgrow the pathogen
1.	NRCL T-01	*Trichoderma viride*	20.7	70.5	8	34.7	60.9	6
2.	NRCL T-02	*Trichoderma harzianum*	31.7	54.8	10	47.3	46.6	9
3.	NRCL T-03	*Trichoderma harzianum*	31.3	55.2	12	49.7	44.0	11
4.	NRCL T-04	*Trichoderma viride*	33.0	52.9	11	46.3	47.7	9
5.	NRCL T-05	*Trichoderma viride*	36.3	48.1	14	47.0	47.0	12
6.	NRCL T-06	*Trichoderma viride*	39.7	43.3	12	50.0	43.6	12
7.	NRCL T-07	*Trichoderma viride*	32.3	53.8	10	47.7	46.2	9
8.	NRCL T-08	*Trichoderma virens*	30.0	57.1	13	47.7	46.2	10
9.	NRCL T-09	*Trichoderma pseudokoningii*	41.0	41.4	14	49.7	44.0	12
	NRCG T-09	*Trichoderma viride*	40.0	42.9	14	52.3	41.0	11
	Control (C)	-	70.0	0.0		88.7	0.0	
	LSD ($p<0.05$)		2.72			2.57	60.9	
	SEm (±)		0.92			0.87	46.6	

C= Radial growth of pathogen in control plate (monoculture), T = Radial growth of pathogen in dual culture with *Trichoderma* sp., PIRG= Percent inhibition of radial growth; * Mean radial growth after 6 days of incubation. NRCG T-09 obtained from Directorate of Groundnut Research, Junagadh, Gujarat was used as reference strain for comparison.

Table.2 Effect of different pH on radial mycelia growth of the two isolates of *Trichoderma viride* incubated at 28 °C

pH	Mean radial mycelia growth (cm)					
	After 24 hr		After 48 hr		After 72 hr	
	NRCL T-01	NRCG T-09	NRCL T-01	NRCG T-09	NRCL T-01	NRCG T-09
4.0	1.92	1.37	5.70	4.88	8.50	7.63
5.0	1.72	1.18	5.40	4.87	8.70	7.70
6.0	1.95	1.88	4.93	5.97	8.87	8.49
7.0	2.02	1.58	5.72	5.73	8.97	8.50
8.0	1.57	1.56	4.90	5.25	8.73	8.47
9.0	1.38	1.54	5.03	5.47	8.67	7.70
LSD (p<0.05)	0.27	0.30	0.46	0.70	NS	0.52
SEm (±)	0.09	0.10	0.15	0.23	0.10	0.17

*NS= Non-significant

Table.3 Effect of application of *Trichoderma viride* on litchi trees affected by wilt and its population dynamics at NRCL Experimental Farm, Muzaffarpur during 2015-2017

Tree no.	Age of the tree	Cultivar	Tree conditions before application	Time taken for complete recovery (days)	Population of *Trichoderma* sp. in rhizosphere (10^3 cfu /g of soil)			
					0 day	15 day	30 day	45 day
1	10	'Shahi'	Yellowing and drooping of leaves	25	6.67	19.33	38.00	27.91
2	15	'Shahi'	Tree decline, yellowing and drooping of leaves	30	4.30	15.07	37.33	28.83
3	5	'China'	Tree decline	25	5.00	13.02	30.99	27.91
4	6	'Shahi'	Early symptoms of tree wilting	20	5.63	15.99	32.86	28.83
5	5	'China'	Early symptoms of tree wilting	20	5.86	25.81	34.97	30.99
6	13	'Shahi'	Tree decline, partial wilting	35	4.90	23.16	42.86	35.33
7	2	'China'	Yellowing and drooping of leaves, stunted growth	20	6.47	18.62	34.97	27.91
8	4	'Shahi'	Yellowing and drooping of leaves, stunted growth	20	2.10	22.38	60.53	48.83
9	6	'Shahi'	Early symptoms of tree wilting	25	5.07	19.33	37.91	30.99
10	12	'Shahi'	Tree decline, partial wilting	30	3.67	19.67	35.33	32.86

Pathogen inhibition through non-volatile metabolites of *Trichoderma*

Production of non-volatile compounds inhibitory to pathogens is one of the multiple modes of action of *Trichoderma* spp. *In vitro* antagonistic activity of culture filtrates of isolate NRCL T-01 significantly and variably reduced the radial colony growth of test pathogen.

It was observed that all concentration of culture filtrates had strong inhibitory effect but the highest inhibition of pathogen was observed at a concentration of 75% liquid culture filtrate where the isolate NRCL T-01 could inhibit growth of *A. alternata* up to 74.7% (radial mycelia growth in treatment 2.03 cm in contrast to 8.03 cm in control) and 37.71 % of growth of *Fusarium solani* (radial mycelia growth in treatment 5.12 cm in contrast to 8.22 cm in control) (Fig. 5 and 6). No further increase in growth of the pathogen was observed till 15th day of incubation.

In contrast to this, the isolate NRCG T-09 could inhibit growth of *A. alternata* up to 43.58% (radial mycelia growth in treatment 4.53 cm in contrast to 8.03 cm in control) and 30.29 % of growth of *Fusarium solani* (radial mycelia growth in treatment 5.73 cm in contrast to 8.22 cm in control) (Fig. 7 and 8).

Our results corroborate the findings of Waseem *et al.,* (2013) who reported that non-volatile antifungal compounds extracted from the liquid culture of *Trichoderma* strain SQRT037 significantly inhibited the growth of *F. oxysporum*. f. sp. niveum which causes wilt of watermelon.

The experimental results of these studies *viz.* dual culture assay, production of volatile and non-volatile metabolites by the indigenous local isolate of *Trichoderma viride* NRCL T-01 thus have proved its antagonistic potential. This was further tested for tolerance to different temperature regime, pH and salt concentrations to assess its biological fitness under *in-vitro* conditions.

Tolerance to temperature

The *T. viride* isolate NRCL T-01 exhibited both high and low temperature tolerance though the radial mycelia growth rate varied at different temperature regimes. Studies showed that after four days of incubation at 15 °C, radial mycelia growth was 9.0 cm at 28 °C while it was only 4.27 cm at 15 °C and 4.23ºC at 40°C (Fig. 9). The highest growth rate was observed at 28 °C (overall growth rate 1.29 cm per day) and lowest at 40°C (overall growth rate 1.15 cm per day). Both increase and decrease in temperature from 28°C resulted in a decrease in radial mycelia growth. No colony growth was observed at 45°C. Poosapati *et al.*, (2014) reported that *T. asperellum*, TaDOR673 was able to survive and germinate normally at 28°C and survived through adverse temperature stress conditions. Petrisor *et al.*, (2016) also reported that growth of *T. viride* strain Td50 was better at 25-30 °C and very slow at 15 °C.

Tolerance to pH

Results indicated that both the isolates of *T. viride* developed at all the pH examined (4.0-7.0) however, variation in radial mycelia growth was apparent between isolates and between pH values. Observations made at 24 hr interval after incubation at 28±1 °C revealed that growth of both the isolates were significantly higher between pH 6.0-7.0 (Table 2). This result corroborates with the findings of Bitton and Boylan (1985) and Limón *et al.*, (2004) who reported that acidic pH favoured fungal growth than alkaline pH and they modify the rhizosphere soil by acidifying the soil. This explains the reason for isolates which prefer acidic pH. However,

our isolate of *T. viri*de, NRCL T-01 could grow well in a wide range of pH, thus indicating its wider adaptability.

Tolerance to salt concentration

The radial mycelia growth of the isolate NRCL T-01 decreased significantly with increase in salt concentration in the PDA medium. After 3rd day of incubation, the highest growth reduction (88.9%) was apparent in 1.5 M salt concentration compared to control plate (Fig. 10) followed by 1.25 M (86.9%), 1.00 M (74.7%), 0.75 M (41.6%), 0.50 M (30.6%) and 0.25 M (19.0%) salt concentration added to the medium. Leo Daniel *et al.,* (2011) also reported similar results while characterizing *T. viride* for abiotic stress. Besides decrease in rate of growth, salt concentration in medium (salinity stress) also resulted in distinct changes in morphology, and a gradual reduction in sporulation with increasing concentration (Fig. 11) was observed. Knowledge of salinity tolerance and its potential effects on the antagonist will be helpful for disease management strategies.

Effect of application of *Trichoderma viride* in controlling litchi wilt

A talc based formulation of *Trichoderma viride* isolate NRCL T-01 was developed with minimum count in final product 2×10^6 cfu/g. The results showed that all the potted plants having inoculation of only *F. solani* wilted in 20-30 days post inoculation, while the plants having application of talc based formulation of *Trichoderma viride* isolate NRCL T-01@ 50 g/plant plus *F. solani* did not wilt indicating the efficacy of the isolate to manage litchi wilt caused by *F. solani*. From the rhizosphere soil samples and fine root bits of wilted plants, *F. solani* was isolated on PDA medium that proves cause of wilting as inoculation with *F. solani.*

The results of the field evaluation of the *T. viride* isolate NRCL T-01 presented in Table 3 demonstrated that it effectively controlled the wilt pathogen and trees recovered in 20-35 day depending on the age and initial condition of the tree. Application of *Trichoderma* formulation resulted in not only 100% recovery of affected trees but also the trees regained vigour and returned to normal health (Fig. 12). The population dynamics of *Trichoderma* sp. in tree rhizosphere showed that at the time of application, the total count of viable prapagules were $2.10\text{-}6.67\times10^3$ cfu/g soils which increased up to 30 days of application ($30.99\text{-}60.53\times10^3$ cfu/g soils). It is probably an important character in quick colonization of rhizosphere possibly because of the hydrolysed organic matters. This is the first report of use of *Trichoderma* to manage wilt caused by *F. solani* in litchi.

In conclusion, the results of the study presented in this paper is the first step in developing *T. viride* isolate NRCL T-01 as a biological control agent for managing litchi diseases especially wilt that affect trees during early establishment phase. The *in vitro* screening as dual culture assay showed consistent antagonism of the isolate NRCL T-01 against *A. alternata* and *F. solani*. The other parameters studied such production of volatile and non-volatile inhibitors; temperature, pH, and salt tolerance establish its fitness as a candidate biocontrol agent. The glasshouse and field studies proved its efficacy in managing wilt disease of litchi caused by *F. solani.* Additionally, the isolate showed good plant growth promotion activity acting as a biofertilizer and helping air-layers to establish better in field (data not provided). Further testing has begun to validate the potential as a commercially-viable product under farmers' field conditions. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the

existing chemical treatment methods. Hence, *Trichoderma* spp. are now the most common fungal biological control agents that have been comprehensively researched and deployed throughout the world.

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