https://doi.org/10.56093/ijas.v90i5.104387

Indian Journal of Agricultural Sciences **90** (5): 1020–4, May 2020/Article

Exploiting plant growth promoting *Trichoderma* for lentil wilt management

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Received: 19 May 2019; Accepted: 31 August 2019

ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lentis* (Fol) has been considered as destructive seed and soil-borne disease of lentil which causes severe yield loss every year. For its biological control, twenty isolates of *Trichoderma* spp. were tested against thirty Fol isolates using dual culture technique. Out of twenty *Trichoderma* isolates, seven isolates (5593, ThL-1, ThL-4, TaL- 2, TaL- 4, ThaL-3 and TvL-6) showed maximum percent inhibition of radial growth against pathogens between 82.4% to 78.0% as compared to the rest of *Trichoderma* isolates. As far as molecular identification is concerned the effective six isolates after dual culture assay and highly virulent Fol isolate were molecularly identified by using ITS primers (ITS 1 and ITS 4) and remaining one isolate was taken from ITCC which was already identified as *T. harzianum* (5593). Seven efficient isolates of *Trichoderma* were used to evaluate volatile and non- volatile assay against Fol (NFLRJ-3). Isolates, 5593 and ThL-4 showed significant growth inhibition against Fol at 48 hours of incubation in both the assays as compared to the rest *Trichoderma* isolates. In volatile organic compound (VOC) assay ThL-4 showed highest number of seed germination and showed good seed health condition. Out of seven selected isolates of *Trichoderma,* all isolates were found effective to control disease and stimulated plant growth promotion activity of lentil (2017-2019). Therefore, these isolates can be utilized to formulate bio-fungicides which is the need of the hour as far as disease management is concerned.

Key words: Biocontrol agent, Mycoparasitism, Scanning Electron Microscopy, Seed germination percentage

Lentil (*Lens culinaris* [L.] Medik) or masoor is a winter season pulse crop (Pérez de la Vega *et al.* 2011) and acts as a major source of dietary proteins (25%) after soybean in human and animal diet of the world (Narayan and Kumar 2015). India is the largest producer, importer and consumer of pulses in the world and accounts for 25% of production, 15% trade and 27% consumption (Narayan and Kumar 2015). Unlike other cereals and commercial crops, the pulse crops are highly risk prone and are vulnerable to diseases, pests attack and sensitive to weather. The per capita availability of pulses per day has declined sharply over a period of time from 60.10 g in 1951 to 35.50 g in 2011 (Tiwari and Shivhare 2016). Among biotic stress, *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *lentis* (Fol) is one of the most problematic disease of lentil worldwide. This disease either individually or in association with other pathogens play significant role in causing low yields in lentil. The wilt of lentil can be overcome through use of resistant cultivars, the varieties with resistant or tolerant to wilt have been bred but the reactions of these varieties against wilt is different under different agro-ecological niches (Naimuddin and Chaudhary 2009). Chemical fungicides cause environmental damage to the soil and heavy metals can accumulate in the human body, which are carcinogenic. Fumigation of soil before planting with MBr (methyl bromide) is an expensive, hazardous and producing ozone-depleting compound. Toxins of chemical fungicides remain in soil for years and contaminate the environment. Therefore, instead of using chemical fungicide, biological control is the good alternative of controlling the pathogen. Plant growth promoting *Trichoderma* is used as promising biocontrol agent (BCA) for management of wide range of crop (Saba *et al.* 2014). The main objective of the present investigation was to evaluate and compare the efficacy of *Trichoderma* isolates against seed and soil-borne pathogen of Fusarium wilt, i.e. *F. oxysporum* f. sp. *lentis* (Fol) *in-vitro* as well as under *in-vivo* conditions.

MATERIALS AND METHODS

Collection and isolation of Trichoderma and Fol isolates

Lentil rhizosphere soil samples were collected from different fields of UP, Delhi, Haryana and Rajasthan during 2016- 2017 and were used for the isolation of *Trichoderma* and *Fusarium* isolates on *Trichoderma* Selective Medium (TSH) and Selective *Fusarium* Agar (SFA) respectively by using serial dilution technique (Leslie and Summerell 2006).

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The appearance of colonies was recorded from 3 to 5 days of inoculation and identification was done by compound microscope. Individual colonies were maintained in PDA slants at 4°C for subsequent studies. Seven *Trichoderma* isolates (5593, 7160, 7172, 7272, 7848, 7856 and 7885) were procured from ITCC (Division of Plant Pathology, IARI, New Delhi) and twelve Fol isolates (FLMP-30, FLMP-87, FLMP-75, FLJH-2, FLJH-5, FLJH-10 (P), FLUP-22, FLCG-1 (P), FLDL-1, FLDL-1 (P), FLBR-13, FLRJ-1) were procured from pulse laboratory (Division of Plant Pathology, IARI, New Delhi). Thus, a total of 20 *Trichoderma* isolates and thirty Fol isolates were used in the present investigation.

Pathogenicity

Pathogenicity of thirty Fol isolates were checked on highly susceptible cultivars of lentil namely Vidhokar Local in a net house during rabi season in 2016-2017 and 2017–2018.All thirty isolates of Fol were analysed for their virulence by following the protocol used by Dubey *et al.* (2012).

DNA isolation and molecular characterization

Genomic DNA was extracted using Allprep® fungal DNA isolation kit (Qiagen). Quantified DNA were stored at −20 °C until further use. Unidentified *six Trichoderma and highly pathogenic Fol isolate* were characterized molecularly based on conserved ribosomal internal transcribed spacer (ITS) region using universal primer pairs ITS1 and ITS4. Amplification was performed on a Thermal Cycler (Biorad; C-1000 Touch) and were confirmed by 1.2% agarose gel electrophoresis (Kumar and Shukla 2005).

Mycoparasitism

Dual culture interaction between most virulent isolate of Fol and all *Trichoderma* isolates were observed using dual culture technique on PDA (HiMedia) (Morton and Strouvle 1955). The plates were incubated at 28 ± 2°C. The growth of the pathogen in both test and control experiments were recorded after 4^{th}, 7^{th} and 14^{th} days of inoculation for percent inhibition of mycelium growth (PIMG) = (R1–R2)/R1 ×100. Where R1 = radial growth of pathogen in control, R2 = radial growth of pathogen in dual culture experiments with antagonists.

Volatile and non-volatile assay

Selected seven *Trichoderma* isolates after dual culture assay were further studied for their volatile and non-volatile metabolites activity by following the method of Dennis and Webster (1971). The same seven *Trichoderma* isolates were also inoculated in 100 ml sterilized potato dextrose broth separately and after 21 days of incubation, culture filtrates were centrifuged and sterilized by using 0.22 µm cellulose acetate and cellulose nitrate membrane vacuum filter (MF-Millipore). After 4^{th}, 7^{th} and 14^{th} days of incubation at 28±2°C, radial mycelial growth of the pathogen was recorded and inhibition percentage of mycelial growth was calculated as described above in dual culture method.

Detection of mycoparasitic interaction of Trichoderma spp. against Fol using SEM

Mycoparasitic interaction of *Trichoderma* isolate against Fol were determined and the interaction was captured by using Scanning Electron Microscope (ZEISS: EVO/MA10) at Division of Entomology, IARI, New Delhi. The preparation of sample was performed by method described by Kinden and Brown (1975).

Volatile organic compound analysis for seed germination activity of Trichoderma on lentil seeds

To analyse VOC of seven *Trichoderma* isolates, tripartite plate method with activated charcoal as volatile trapper was used for their seed germination activity on lentil seeds.

In vivo analysis of plant growth promotion efficiency and biocontrol activity of Trichoderma sp. isolates

To evaluate the growth promotion and biocontrol efficacy of *Trichoderma* isolates on lentil against *Fusarium* wilt, field trial was conducted at Vegetable field of ICAR-IARI, New Delhi during rabi season in 2017-2018 and 2018- 2019. The experiment was conducted in a Completely Randomized Block Design with three replications.

RESULTS AND DISCUSSIONS

Isolation of Trichoderma and Fol isolates

Out of 20 *Trichoderma* and 30 Fol isolates, 13 *Trichoderma* and 18 Fol isolates were isolated from rhizosphere soil collected from different regions of lentil crop, while rest others were collected from Division of Plant Pathology, IARI, New Delhi.

Pathogenicity test of Fol under pot trial

All the thirty isolates of Fol caused wilt and their severity varied based on virulence of the isolate. The most virulent one isolate was NFLRJ-3 (isolated from Rajasthan soil sample) as compared to other isolates which were less or moderately virulent. When re-isolation was carried out from (inoculated) infected plants, Fol was observed which confirmed their pathogenicity. Similarly, Kumar *et al.* (2011) have carried out the pathogenicity test on chickpea against *Fusarium oxysporum* f. sp. *ciceris*.

Molecular identification based on ITS

The ITS rDNA sequences of approximately 530 nucleotides homologies were examined by comparing to NCB using BLASTN and submitted to NCBI GenBank which are as follows: two isolates of *Trichoderma harzianum* (ThL-1 isolated from Rajasthan accession no.: MK300029.1 and ThL-4 isolated from UP, accession no.: MK300031.1), two isolates of *Trichoderma asperellum* (TaL-2 isolated from UP, accession no.: MK300032.1 and TaL-6 isolated from Rajasthan, accession no.: MK300034.1), *Trichoderma* *hamatum* (ThaL-3 isolated from Haryana, accession no.: MK300030.1), *Trichoderma virens* (TvL-5 isolated from UP, accession no.: MK300033.1) and *Fusarium oxysporum* f. sp. *lentis* (NFLRJ-3 isolated from Rajasthan, accession no.: MK300027.1).

Dual culture assay against F. oxysporum isolates by Trichoderma isolates

Trichoderma isolates were evaluated for their mycoparasitic activity against Fol (NFLRJ-3) which showed significant decrease in terms of radial mycelium diameter after treatment, as compared with the control. Out of 20 *Trichoderma* isolates, seven isolates (5593, ThL-1, ThL-4, TaL- 2, TaL- 6, ThaL-3 and TvL-5) showed maximum radial growth percent inhibition of Fol between 98.4% to 80.0% as compared to the rest of *Trichoderma* isolates which showed radial growth inhibition less than 75%.

Volatile and non-volatile assay

Among seven highly efficient *Trichoderma* isolates (after dual culture assay), volatile metabolites of 5593 and ThL-4 showed significant growth inhibition against Fol at 48 hours of incubation. The inhibition produced by the 5593 and ThL-4 (67.5% and 65.0%) were significantly higher than that produced by other isolates of *Trichoderma* (Table 1). The volatile compounds of *T. atroviride* and *T. harzianum* significantly reduced the growth of *Cytospora chrysosperma* and *Dothiorella gregaria* (Gao *et al.* 2001). Non-volatile metabolites of *Trichoderma* spp. showed that the products of 5593 and ThL-4 isolates completely suppressed the growth of Fol and almost 100 percentage of inhibition at 48 hr of incubation at 35% v/v concentration. On the other hand, rest *Trichoderma* isolates had significantly lower inhibition on the tested pathogen as compared to 5593 and ThL-4 (Table 1). Similar results were found by Deshmukh and Pant (1992) that the non-volatile culture filtrate of *Trichoderma* sp. reduced the mycelial growth of *Fusarium* spp.

Determination of lentil seed germination analysis of Trichoderma by using tripartite plates

The demonstration of volatile organic compounds (VOC) produced by all *Trichoderma* isolates on the seed germination of lentil was determined in this experiment where, out of seven *Trichoderma* isolates 5593, ThL-4 and ThL-1 (Table 2) were found more effective for seed germination activity as compared to others.

Detection of mycoparasitic interaction of Trichoderma spp. against Fusarium oxysporum using SEM

Mycelial samples collected from the interaction zone

Table 1 Percent inhibition of volatile and non- volatile compound of *Trichoderma* isolates against Fol (NFLRJ-3)

Trichoderma isolates	Volatile assay		Non-volatile assay			
	Radial growth (cm) of pathogen	Percent inhibition of pathogen	Percent of inhibition of *Fol* at different concentration of Non-volatile compound of *Trichoderma* isolates			
			15%	25%	35%	45%
5593	2.6	67.5	65.0	63.9	100.0	100.0
ThL1	3.5	56.2	51.7	70.5	90.5	100.0
TaL-2	3.9	51.2	32.8	72.5	63.9	82.5
ThaL-3	4.3	46.2	35.2	63.9	68.5	85.0
ThL-4	2.8	65.0	63.9	37.5	99.2	100.0
TvL-5	4.0	50.0	18.2	62.3	69.8	80.0
TaL6	3.8	52.5	55.9	69.0	75.5	79.7
Control (radial growth in cm)	8.0	-	8.0			

Table 2 Seed germination percentage of *Trichoderma* isolates with and without activated charcoal

Entry	Ac + T + S		T + S		AC + S	
	Percent of seed germination	Seed germination length (cm)	Percent of seed germination	Seed germination length (cm)	Percent of seed germination	Seed germination length (cm)
5595	90	1.08	98	2.82	90	1.25
ThL1	90	1.28	94	2.50	92	1.75
TaL-2	90	1.20	92	2.20	90	1.62
ThaL-3	85	1.10	89	1.89	90	1.46
ThL-4	89	1.35	96	2.73	91	1.81
TvL-5	87	1.35	91	2.02	89	1.50
TaL6	90	1.20	94	2.35	91	1.15

AC: Activated charcoal; T: *Trichoderma* isolate; S: Lentil seeds

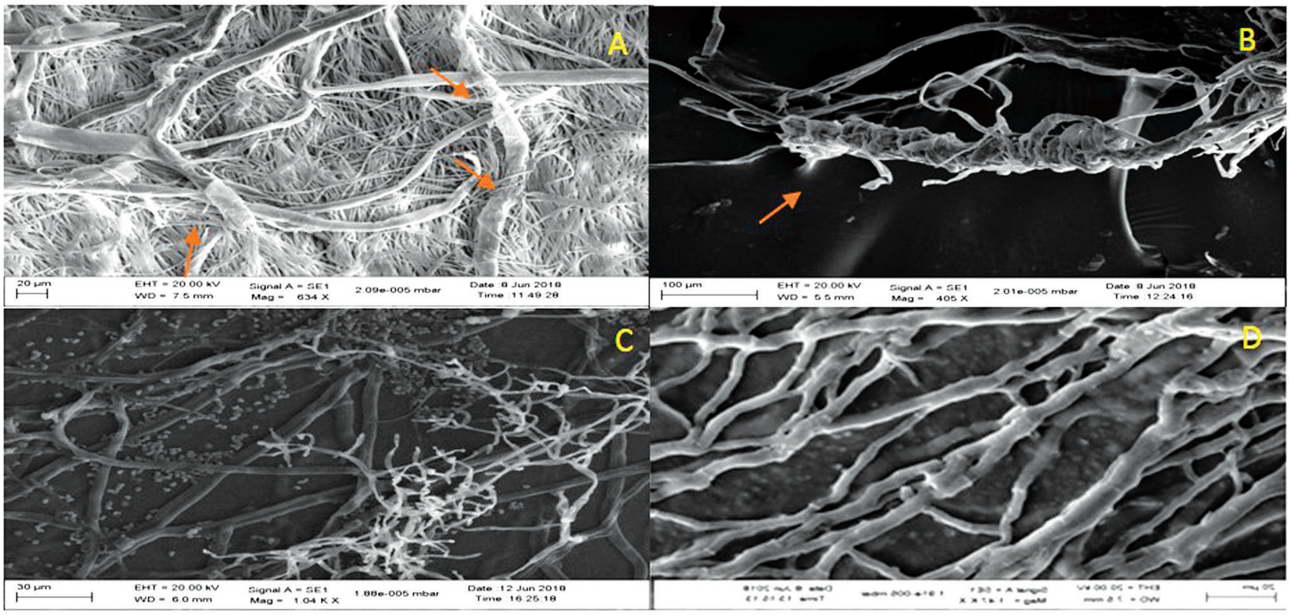

Fig 1 Mycoparasitism activity of *Trichoderma* isolates against Fol via Scanning Electron Microscopic (SEM).

of *Trichoderma* isolates and NFLRL-3 were observed. The antagonist, *T. harzianum* was most often distinguished from Fol by hyphal diameter (Fig 1: where, *A and B: S*upercoil of *Trichoderma isolates* on *Fusarium mycelium C: Trichoderma*ThL-4 *mycelium* and D: Mycelia of Fol). Indeed, the average diameter of *T. harzianum* hyphae was estimated to be 2µm, whereas those of Fol hyphae ranged between 3 and 6µm. At an early state of parasitism (2 days after inoculation), *T. harzianum* hyphae established close contact with the host by coiling around the hyphae. The coils were usually very dense and appeared to tighten the hyphae of Fol. However, at this stage of the interaction, integrity of cell surface of the host was well preserved. The interactions observed in the SEM analysis can be considered as typical of hyperparasitism (Agrios 2005). Similarly, Louzada *et al.* (2009) observed hyphae of *F.solani* encoiled by *Trichoderma* in SEM images. As reported by Zeilinger and Omann (2007) during mycoparasitism lectines from the cell wall of the pathogen can induce the association of antagonist hyphae with the pathogen and consequently hyphae of the pathogen may be colonized.

In vivo analysis of plant growth promotion efficiency and antagonistic activity

Out of seven selected isolates of *Trichoderma,* all isolates were found effective to control disease and also stimulate plant growth promotion activity of lentil seeds. The two isolates namely 5593 and ThL-4 were found to be most expensive in relation to plant growth promotion activity and antagonistic activity under *in-vivo* condition (Table 3). Previous research has also shown that *Trichoderma* promotes plant root development and solubilize nutrients (Contreras-Cornejo *et al.* 2009). The ability of *Trichoderma* to increase tomato biomass in the absence of the pathogen suggests that these isolates are likely to be able to influence the production of phyto-

Table 3 PGP activity and inhibition of diseases by *Trichoderma* isolates under field condition (mean data of two year 2017- 2018 & 2018-2019)

Trichoderma isolates	No. of plants		Percent of wilt		Fresh weight /plant (g)		Dry weight / plant (g)		Branches/ plant		Root size (cm)/ plant		Shoot size (cm)/ plant		Yield in kg/ plot	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
5593	91	89	0	4	35.5	32.5	24.5	22.4	6	5	13.9	12.0	20.6	27.5	1.26	1.18
ThL1	85	86	1	8	31.5	31.3	21.0	21.7	3	4	12.0	11.7	24.7	22.4	1.19	1.09
TaL-2	79	74	0	9	30.0	18.8	14.0	12.6	3	3	10.5	9.0	18.3	17.9	1.13	1.04
ThaL-3	76	69	8	12	22.6	20.9	18	15.0	3	3	8.9	9.2	16.9	18.2	1.11	1.03
ThL-4	94	90	0	7	33.3	30.0	23.1	21.7	5	5	12.5	11.6	25.4	23.6	1.24	1.17
TvL-5	78	64	6	12	28.0	25.0	19.6	17.5	3	3	9.7	10.5	21.0	18.3	1.06	0.99
TaL6	82	70	3	5	30.2	25.5	19.0	15.5	3	3	11.0	10.3	18.5	18.9	1.12	1.01
Control	70	62	10	23	20.7	18.8	14.0	12.6	3	3	8.9	8.5	18.3	17.9	1.02	0.85

R- Resistant cultivar of lentil L-4147; S- Susceptible cultivar of lentil Vidhohar Local.

stimulators or phytohormones (Martínez-Medina *et al.* 2014). Kumar *et al.* (2016) have observed enhanced plant growth promotion activity and antagonistic activity of pathogen by biaogent in combination mode.

Microorganisms that grow in the rhizosphere are ideal for use as biocontrol agents. Our studies proved that two isolates of *Trichoderma harzianum* namely 5593 and ThL-4 have the potential to control Fol *in vitro* as well as under *in vivo* condition and also stimulate PGP activity in lentil. Therefore, these isolates can be utilized to formulate bio-fungicides, which is the need of the hour as far as disease management is concerned.

ACKNOWLEDGEMENTS

Authors are thankful to SERB funding agency, Division of Seed Science and Technology, ICAR-IARI, Dr N Srinivasa, Division of Plant Pathology, ICAR-IARI. This research work is part of the Ph D programme. Hence, the first author is thankful to the Director and Professor of the Amity Institute of Microbial Technology, Amity University, Noida for supporting in the research work.

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