IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 10, Issue 10 Ver. I (October 2017), PP 47-52
www.iosrjournals.org

Isolation, characterization and growth on Agri Residues of a new Isolate *Trichoderma asperellum* RSTV04 useful in inhibiting Tomato wilt causing *Fusarium oxysporum*

G. Babul Reddy[1]*, Karuganti Sukumar[1], K.Ravi Kumar[2], G. Swarnabala[2], G.Venkateswarlu[2], K. Venkatanand Reddy[2],V.Muneel[2]
RADOSOM, R&D center, Som Phytopharma(India) Ltd, Hyderabad, India
**Corresponding author:*
Email ID: babulreddy@agrilife.in

Abstract: *Fusarium oxysporum was isolated from the wilt affected tomato plants in a farm in Sadasivapet, Telangana by characterization and morphological properties. Several Trichoderma spp. (*RSTV01, RSTV02, RSTV03, RSTV04 and RSTV05*) were isolated from agricultural lands in the vicinity of Sangareddy and antagonistic activity of these isolates was studied on the above isolated Fusarium oxysporum. Of all these 5 Strains* RSTV04 *was found to have good inhibition on growth of Fusarium oxysporum. 18S ribosomal RNA sequencing was done for* RSTV04 *and was identified as Trichoderma asperellum. In present study, different cost effective agricultural residues and additives were used for the mass production of Trichoderma asperellum through solid state fermentation. Among all these different substrates and additives Maize Grit had shown higher activity among all the substrates with 8.9x10^9/g of Koji followed by Wheat bran with 1.8x10^9/g of Koji with Jaggery as an additive.*

Keywords: *Antagonistic activity, Different agricultural residues, Fusarium oxysporum, Tomato wilt, Trichoderma asperellum.*

Date of Submission: 30-09-2017 Date of acceptance: 14-10-2017

I. Introduction

After green revolution, agricultural techniques are intensified to meet the demands of food and fiber all over the world, by using Synthetic fertilizers and other Chemicals. Due to this the natural ecosystem is getting damaged by polluting environment, ground water and food materials. Globally plant diseases are considered as the major problem as they are causing high loss in the agriculture [1].Different Bacteria, Fungi, Protozoa, Viruses and Pests cause several diseases in various developmental stages of the plants. In order to prevent these plant diseases chemical pesticides are mostly used [2]**.**

The usage of Chemical pesticides is showing adverse effects on the environment and non-target organisms. The harmful effects of the Chemical pesticides and fertilizers warrant an eco-friendly pest management system. Excessive use of the Chemical fungicides resulted in the deposition of toxic compounds which are harmful to the humans, non-target animals, and environment. It also resulted in developing resistance of the plant pathogens [3]**.**An alternative to the existing chemical treatment methods is the Biological control of plant pathogens by using beneficial microbes which are natural and environmentally safe [4].

Since 1930's, *Trichoderma* has been extensively used to control the plant diseases because of its antifungal activity [5]. *Trichoderma* genus has the capability of producing cell wall degrading enzymes such as Cellulase, Hemicellulase, Xylanase, Chitinase, β-glucanases and Secondary metabolites which are involved in biocontrol activity, but among all these enzymes Cellulases play a major role in controlling the plant pathogenic fungi [6] [7]. In *Trichoderma* genus, *Trichoderma asperellum* is also an antagonistic fungus having regularly branched and paired conidiophores with an optimum growth temperature of 30^{0}C [8].

Fusarium oxysporum is a soil borne pathogen which causes Vascular wilt disease in all the Horticultural crops. *F.oxysporum* produces a specialized structure known as *formae specialis* by which it infects different commercial crops such as Banana, Cabbage, Cotton, Watermelon, Tomato *etc.* [9]**.** *Trichoderma* species are found to be effective biocontrol agents against wide range of plant pathogens including *Fusarium* species [10].

Mass production of *Trichoderma asperellum* can be done by using Submerged (Smf) and as well Solid state fermentation (SSF). Recently SSF technique has been used mostly as it is natural, cost effective and simple technique when compared to Smf [11].As the chemicals used in the synthetic medium are very costly, and the

residues of medium after the fermentation show toxic effect on the environment. In this study, they are replaced with various agricultural and industrial residues which can be used as substrates in the Solid state fermentation.

Selection of substrate plays a major role in the mass production of *Trichoderma*. Substrate selected should be readily available, cost effective and stable [12]. Some of the factors which play a major role in SSF are Inoculum percentage, Surface area, Particle size of the substrate, Moisture, Aeration, pH, and Temperature [11]. In the present study for the mass production of *Trichoderma asperellum* various agricultural residues were used in order to arrive the most suitable ones, which are cost effective and yield higher spore count.

II. Materials and Methods

2.1.Isolation and identification of *Trichoderma spp*.

Soil samples were collected aseptically in sterilized containers from different areas of Rejinthal village, Sadasivpeta Mandal, Sangareddy District, Telangana. Samples were serially diluted and *Trichoderma* selective medium (TSM) was used for the isolation.

The basal medium consisted of the following ingredients/L: 0.2 g. $MgSO_4$, 0.9 g. K_2HPO_4, 0.15 g. KCl, 1.0 g. NH_4NO_3, 3.0 g. D-Glucose anhydrous, 0.15 g. Rose Bengal and 20.0 g. Agar. These ingredients were mixed in 950 ml of distilled water and sterilized at 121^0C for 30 min. Biocidal components such as 0.25g. Chloramphenicol, 0.2 ml Fludioxonil, 0.02 g. Captan and 1.6 ml metalaxyl were mixed in 50 ml of sterilized distilled water and filter sterilized by using 0.45 microns Nitrocellulose membrane. The biocidal mixture was added to the autoclaved basal medium after cooling it to 40^0- 50^0C [13].

Inoculated plates were incubated at 26^0C for 4 days and the fungal colonies were isolated by using streak plate method. These plates were incubated at 26^0C for 7-8 days for observation of green conidia forming fungal bodies. Isolated pure samples were microscopically observed by using Phase contrast microscope (Nikon Eclipse 200) and the morphology was studied. Pure cultures (RSTV01, RSTV02, RSTV03, RSTV04 and RSTV05) were maintained in Potato Dextrose Agar slants and stored at 4^0C [14]**.** Antagonistic activity of these five strains on *Fusarium oxysporum* was studied and inhibition zone of RSTV04 was found to be more compared to the other 4 strains. Sequencing studies were carried out for the above antagonistic *Trichoderma sp.* RSTV04.

2.2.Isolation of plant pathogen

Samples of diseased tomato plants were collected from Rejinthal village, Sadasivpet, Sangareddy District, Telangana. The diseased fragments were surface sterilized with 3% Sodium hypochlorite for 3 min. The surface sterilized fragments were transferred into Potato Dextrose Agar and incubated at 25^0C for 7 days. Sporulation and pigmentation of the culture were observed [15]**.**

2.3.Antagonistic activity by using Dual culture technique

5mm diameter mycelia disks of *Fusarium oxysporum* were placed on one edge of Potato Dextrose agar plate and incubated at 25^0C for 48 hrs. After the incubation mycelia disks (5mm in diameter) of *Trichoderma spp.* (RSTV01, RSTV02, RSTV03, RSTV04 and RSTV05) were placed on the other side of the Petri plate. Evaluation of radial growth of the pathogen and speed overlapping of *Trichoderma spp.* (RSTV01, RSTV02, RSTV03, RSTV04 and RSTV05) on pathogen were checked in order to know the interactions between these species within 7 days (168 hrs) [16]**.**

Percentage of inhibition was calculated by using the formula [17]:

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

Where

I = Per-cent inhibition in mycelia growth

C = Growth of pathogen in control plates

T = Growth of pathogen in dual culture plates

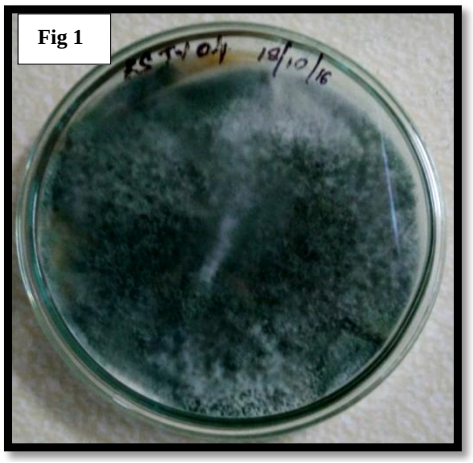
2.4.Mass production by using Solid state fermentation

Different agricultural residues such as Maize grit, Wheat bran, Guar meal korma, Dried distillery grain solids and Chia cake were used for evaluation of best substrate for growing *Trichoderma asperellum*. In addition to the above substrates additives such as Jaggery and Molasses were used. 300 g of above materials were taken in an Autoclavable Polypropylene bag (46 cm x 29.5 cm) with moisture content adjusted to 30 % with Jaggery and Molasses (4% in distilled water) and sterilized at 121^0C for 30 min. Spore suspension of 10 days old *Trichoderma asperellum* RSTV04 culture was made in sterile distilled water. Sterilized substrate bags were inoculated with 10 ml of spore suspension containing $1X\ 10^6$/ ml of spores. After incubating the bags for 10 days the dry spores were harvested by using sieve of 200 µm size. Colony forming units of *Trichoderma asperellum* on different substrates were calculated as per the Standard Plate count protocol [5]**.**

III. Results and discussion

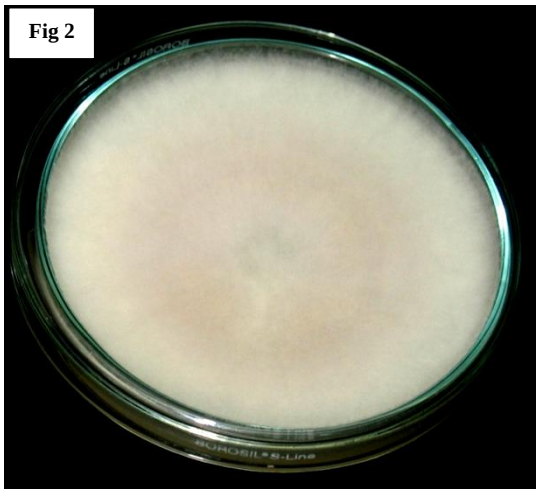
3.1. Screening of *Trichoderma* isolates

Total 5strains were isolated by using *Trichoderma* selective medium and the isolates are named as RSTV01, RSTV02, RSTV03, RSTV04 and RSTV05. Morphology of the cultures and sporulation were studied and microscopic observation was done. Fig 1.



3.2. Isolation and screening of *Fusarium oxysporum*

Root fragments of diseased tomato plants were collected and grown on Potato Dextrose agar. The pathogenic fungus was isolated from the root fragments. Culture morphology, sporulation and pigmentation of the culture were studied on PDA. After the observation of culture characteristics the strain was identified as *Fusarium oxysporum*. Fig.2



3.3. Antagonistic activity

Antagonistic activity/Mycoparasitism of *Trichoderma spp.,* was checked by using Dual culture plate technique. Results of Dual culture plate showed that among all the 5 isolates, RSTV04 showed best mycelial inhibition of *Fusarium oxysporum* with 82.1% against Control Fig 3(a), (b), (c), (d).

Till 7 days Percentage of inhibition was calculated. RSTV04 showed highest inhibition percentage when compared to other isolates Table 1 & Fig.4.

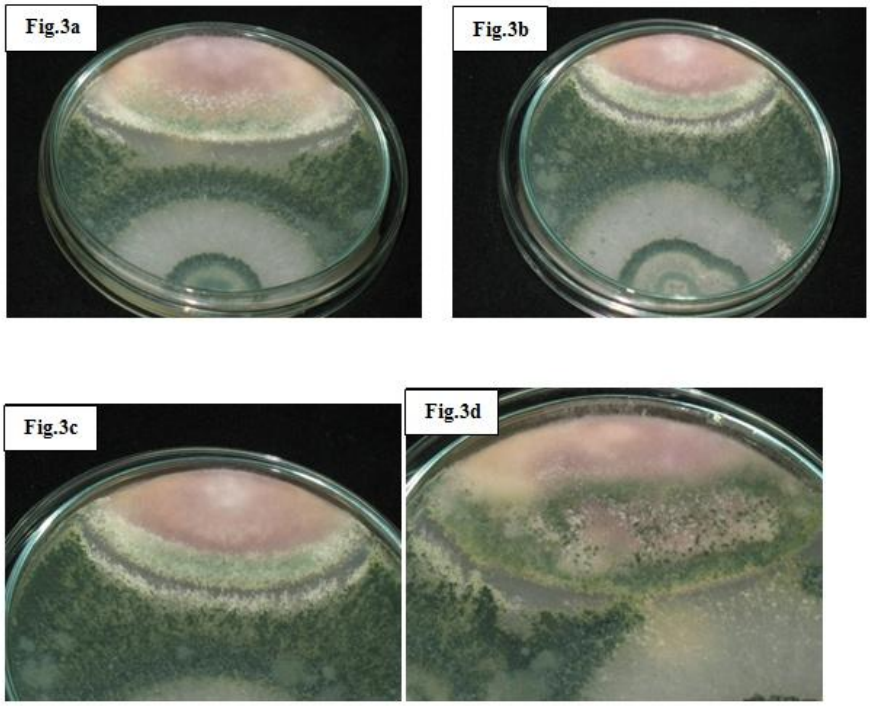


fig 3a – RSTV04 + *Fusarium oxysporum* at 98 h, 3b – After 120 hrs, 3c – After 144 h, 3d – 168 hard copy

Table 1 Percentage of inhibition of RSTV04 strain at regular intervals of time

Time	Percentage of inhibition
96 hrs	52.63 %
120 hrs	65.2%
144 hrs	74.7%
168 hrs	82.1%

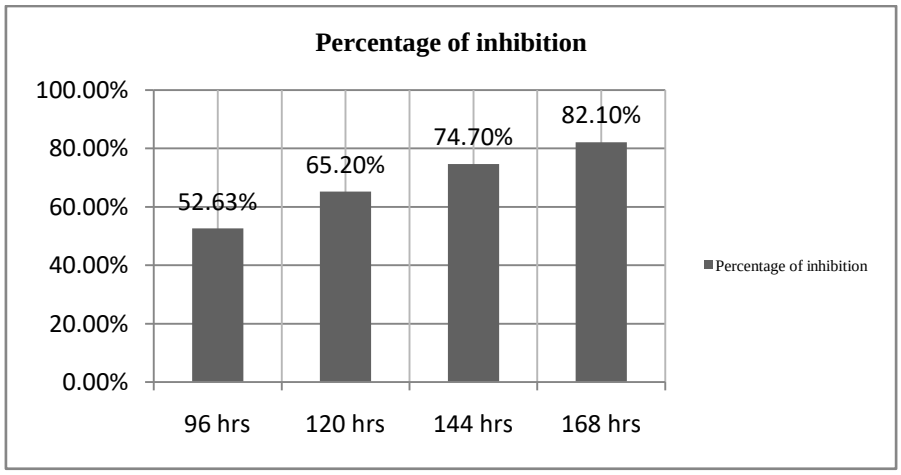


Fig 4: Antagonistic activity of RSTV 04 showing mycelial inhibition percentage till 7 days.

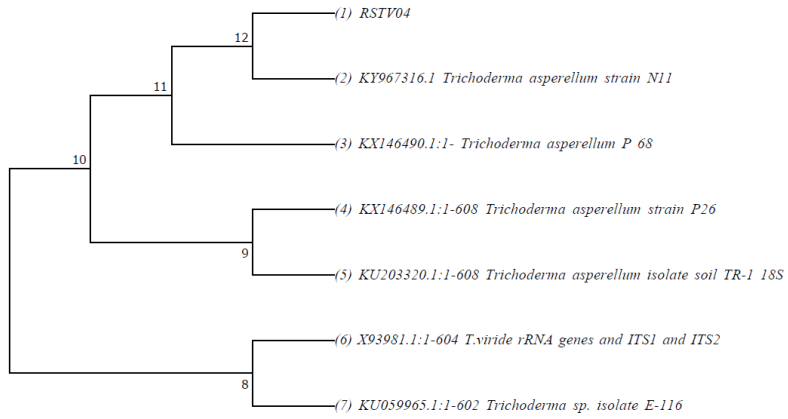
3.4. Genotypic identification of isolated fungal species

Pure culture of RSTV04 was sent to Eurofins Genomics India Pvt Ltd., Bangalore, Karnataka(State), India for molecular identification. Following 18 S r RNA sequence was obtained:

GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAAC
CCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGC
CCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTC
AAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT
GCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGG
TGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAG

CGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGA
ACTTAAGCT

18 S r RNA sequence of RSTV04 was checked in BLAST which is a database of NCBI Genbank and identified as *Trichoderma asperellum.* Maximum identity score of first ten sequences were collected and aligned by using Clustal W. Maximum Likelihood Phylogenetic tree was constructed by using MEGA 7 software.Fig.5



I.

Fig 5: Maximum likelihood phylogenetic tree showing the relatedness of RSTV04 with *T.asperellum*

3.5. Mass production of *Trichoderma asperellum* RSTV04 by using different Agro based residues

Different agricultural residues such as Maize grit, Wheat bran, Guar meal korma Chia cake and Dried distillery grain solids were used as the substrates for mass production of *Trichoderma asperellum*. Moisture content was adjusted by using Jaggery and Molasses.

Among all the substrates Maize grit showed best CFU count $8.9X10^9$/g. of Koji with Jaggery as an additive followed by Wheat bran showing $3.2X10^9$/g. of Koji with Molasses and $1.8X10^9$/g. of Koji with Jaggery. Dried distillery grain solids have shown least CFU count among all the substrates. Growth and sporulation was very quick in Maize grit when compared to the remaining substrates.Table 2.

Table 2: Mass production of *Trichoderma asperellum* RSTV04 by using different agro – based substrates

Sl.no	Substrate	Additives	CFU / g of koji
1	Guar Meal Korma	Molasses	$5X10^7$
		Jaggery	$1.6X10^8$
2	Maize grit	Molasses	$7.1X10^8$
		Jaggery	$8.9X10^9$
3	Chia cake	Molasses	$1.9X10^5$
		Jaggery	$2.8X10^5$
5	Wheat bran	Molasses	$3.2X10^9$
		Jaggery	$1.8X10^9$
6	DDGS (Dried distillery grain solids)	Molasses	Poor growth
		Jaggery	Poor growth

IV. Conclusion

In current study, *Trichoderma asperellum* RSTV04 was isolated from the soil samples and its Mycoparasitism was checked on *Fusarium oxysporum* and has been inhibited the plant pathogen with 82.1% (Percentage of inhibition) within 168 Hrs. under in – vitro conditions. Different agro – based residues and extra additives were taken for the mass production of *Trichoderma asperellum* RSTV04. Among all the substrates selected for the substrate optimization Maize grit had shown high spore count *i.e.* $8.9x10^9$/g of Koji with Jaggery as an additive. As the Maize grit is cost effective and readily available, it can be used for the large-scale production of *Trichoderma asperellum* in Solid state fermentation.

Acknowledgment

We wish to express our gratitude to Dr. Venkatesh Devanur and Dr. G. Vijaya Raghavan for helpful and critical discussions. This work was supported by Som Phytopharma (India) Ltd, Hyderabad.

References

[1] Kumar S, Gupta OM. Expanding dimension of Plant Pathology.*JNKVV Res. J. 46(3)*,2012, 286-293.
[2] Handelsman J, Stabb EV, *Biocontrol of soil borne plant pathogens*, Plant cell 8, 1996,1855 – 1869
[3] Bailey, D.J., A. Kleczkowski and C.A. Gilligan, *New Phytol*., 2004**,**161: 569-576.
[4] S. Shalini, and A. S. Kotasthane, Parasitism of *Rhizoctoniasolani* by strains of *Trichoderma sp*, *EJEAFChe, 6 (8)*, 2007, 2272-2281.
[5] LalSahab Yadav, Antagonistic activity of *Trichoderma sp* and evaluation of various agro wastes for mass production, *Indian Journal of Plant Sciences Vol. 1 (1)*, 2012,109-112.
[6] Sonika Pandey, Mukesh Srivastava, Mohammad Shahid, Vipul Kumar, Anuradha Singh, Shubha Trivedi and Y.K. Srivastava, *Trichoderma species* Cellulases Produced by Solid State Fermentation,*J Data Mining Genomics Proteomics*, 2015, 6: 170.
[7] Abbas A, Jiang D, Fu Y, Trichoderma Spp. as Antagonist of Rhizoctonia solani. *J Plant Pathol Microbiol 8*,2017
[8] Gary J. Samuels, Elke Lieckfeldt&Helgard I. Nirenberg, *Trichoderma asperellum*, a new species with warted conidia, and redescription of *T. viride,1999*.
[9] Mamta Joshi, Rashmi Srivastava, A. K. Sharma and Anil Prakash, Isolation and characterization of *Fusarium oxysporum*, a wilt causing fungus,for its pathogenic and non-pathogenic nature in tomato (*Solanum lycopersicum*),*Journal of Applied and Natural Science 5*, 2013, (1): 108-117.
[10] Somaskhekhara, Y.M., A.L. Siddaramaiah and T.B. Anilkumar, Evaluation of *Trichoderma* isolates and their antifungal extracts as potential biological control agents against pigeon pea wilt pathogen. *Curr. Res*., 1998, 27: 158-160.
[11] Rajshree Saxena, Rajni Singh, Amylase production by Solid state fermentation of Agro- Industrial wastes using *Bacillus sp*, *Brazilian Journal of Microbiology*, 2011, 42: 1334-1342.
[12] Sobita simon and Anamika, Agro-based Waste Products as a Substrate for Mass Production of *Trichoderma* spp, *Journal of Agricultural Science Vol. 3, No. 4*, 2011.
[13] D. J. Askew and M. D. Laing, An adapted selective medium for the quantitative isolation of *Trichoderma species***,** *Ptant Pathotogy* 42, 1993, 686-690.
[14] Mridula Khandelwal, Sakshi Datta, Jitendra Mehta, Ritu Naruka, Komal Makhijani,Gajendra Sharma, Rajesh Kumar and Subhas Chandra, Isolation, characterization & biomass production of *Trichoderma viride*using various agro products- A biocontrol agent, *Advances in Applied Science Research*, 2012, 3 (6):3950-3955.
[15] MajaIgnjatov, DraganaMilošević, ZoricaNikolić, JelicaGvozdanović-Varga,DušicaJovičić and GordanaZdjelar, *Fusarium oxysporum*as Casual Agentof Tomato Wilt and Fruit Rot, *Pestic. Phytomed. (Belgrade)*27(1), 2012, 25–31.
[16] MohammadAkrami, HadiGolzary and Masoud Ahmadzadeh,Evaluation of different combinations of *Trichoderma*species for controlling *Fusarium* rot of lentil, *African Journal of Biotechnology Vol. 10(14)*, 2011, pp. 2653-2658.
[17] Chanchal Gautam and Sharmita Gupta, Antagonistic effect of *Trichoderma viride* and *Trichoderma harzianum* against plant pathogenic fungi and its growth on different agro-waste substrates, *International Journal of Engineering and Technical Research*, 2014, ISSN: 2321-0869.

G. Babul Reddy. "Isolation, characterization and growth on Agri Residues of a new Isolate Trichoderma asperellum RSTV04 useful in inhibiting Tomato wilt causing Fusarium oxysporum." IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS), vol. 10, no. 10, 2017, pp. 47–52.