https://doi.org/10.56093/ijas.v90i6.104754

Indian Journal of Agricultural Sciences **90** (6): 1058–62, June 2020/Article

Biocontrol of *Fusarium* wilt of tomato by nano-extract of *Trichoderma harzianum*

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Received: 5 August 2018; Accepted: 16 September 2019

ABSTRACT

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* is an important disease of tomato worldwide. The pathogen was isolated and purified from infected plants in tomato-growing greenhouses in southwest Iran during 2017. The impact of colony's extract of four *Trichoderma harzianum* isolates were tested on colony growth of the pathogen *in-vitro*. Premium isolate colony's extract of *T. harzianum* was converted to nano-product by mixing with silver-nitrate. The formation of nano-particles in this product was evaluated by dynamic light scattering, and scanning electron microscopy. The impact of nano-product was tested on colony growth of the pathogen and its EC_{50} was determined. Then the impact of this product and silver-nitrate was tested on the disease severity in two tomato varieties *in-vivo*. Colony's extract of all four isolates of *T. harzianum* reduced the colony growth of the pathogen, but the effect of an isolate's extract of *T.harzianum* 39 was more. Produced nano-extract had EC_{50}441.9ppm for the pathogen. It also caused a significant reduction of the disease severity *in-vivo*, and its effect was more than silver-nitrate alone. Potential of *T. harzianum* nano-extract for biological control of the disease is being reported for the first time as per our knowledge.

Key words: Fusarium wilt, Nano, Silver, Tomato, *Trichoderma*

Tomato is the tropical vegetable crop throughout the world. The fruits are rich in vitamin A and C, and are gaining importance because it contains lycopene, a food component known to reduce incidence of prostate cancer, heart and age related diseases (Bawa 2016). Fusarium wilt, caused by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen (*Fol*), is one of the important disease of tomato in the world (Agrios 2005, Reis *et al.* 2005, Ershad 2009). The disease incident with high severity has reported from ten major tomato-growing districts of Tamil Nadu, in India (Amutha and Darwin Christdhas 2017). Also, it is common in central and southern Iran areas, and its damage has been reported up to 27% of the yield (Ershad 2009, Amini *et al.* 2014). Infected bushes are with dark-brown phloem and often become yellow and wilt before flowering (Agrios 2005). Biological control is the best method of disease management, as a safe and secure method for the environment (Bawa 2016). There are several reports of antifungal activity of silver nanoparticles against soil-borne plant pathogenic fungi (Kim *et al.* 2012). Silver particles' antibiotic feature increases up to 99 percent when they converted to the nanometer dimensions. Silver nanoparticles production by using microorganisms is environmentally friendly and economically cheaper. The research works have been conducted to produce the nano-products from extracts of *Trichoderma*, *Penicillium*, *Rhizopus* and *Aspergillus* species (Naveen *et al.* 2010, Banu and Rathod 2011, Roy *et al.* 2013, Shelar and Chavan 2015). *Trichoderma harzianum* Rifai is one of the most important agents of biological control of soil-borne plant pathogenic fungi such as *Fusarium oxysporum* f. sp. *lycopersici* (Mwangi *et al.* 2011, Akrami and Yousefi 2015). It causes their death or growth reduction by producing enzymes and various inhibiting metabolites (Vinale *et al.* 2006). It has been used to biosynthesis silver nanoparticles, and biological control of white mold disease of soybean (Guilger *et al.* 2017). Therefore, this study was conducted to produce the nanoparticles biological fungicide from *T. harzianum* to manage Fusarium wilt of tomato, due to the importance of the disease worldwide.

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MATERIALS AND METHODS

Isolation, purification and identification of the pathogen: The pathogen was isolated and purified from infected plants in tomato-growing greenhouses in southwest Iran, by Amini *et al.* (2014) method (2017). Carnation-leaf-agar (CLA) medium was used for producing macro-conidia. The isolates were identified by studying their morphological characteristics of colonies and spores (Leslie and Summerell 2006).

Pathogenicity test: The seeds of two varieties of tomato's

Rio-grande and Super-strain B were cultured after surface sterilizing with 0.5% sodium hypochlorite for one minute and three times washing with sterilized distilled water, within spatial pots of seedling production in sterilized soil, for pathogenicity test of the pathogen isolates. Twenty-one days seedlings were inoculated by cutting the root tip and dipped in a suspension of 10^7 macro-conidia with each isolate of the pathogen. Pots were maintained in a greenhouse at a temperature of 28°C and a relative humidity of 70% and were irrigated once every three days. Disease severity was determined, based on Sibounnavong *et al.* (2010) scale [1=No symptom, 2=1-20%, 3=21-40%,4=41-60%, 5=61-80% and 6=81-100% of yellowing and wilting of the plant], 60 days after seedlings' inoculation. Data were subjected to analysis of variance (ANOVA) by SAS 9.2 software and treatment means were compared with Duncan's multiple range test (DMRT).

Testing the impact of the colony's extract of T. harzianum isolates on the colony growth of the pathogen isolates in-vitro: Four isolates of *T. harzianum* existed in the collection of fungi in Department of Plant Protection, were selected, and their colony's extract was isolated in potato-dextrose broth (PDB) medium by Shelar and Chavan (2015) method. The effect of obtained extract from each isolate was evaluated by the method of mixing with PDA medium at 6 concentrations of 5, 10, 15, 20, 25 and 35 percent on colony growth of hyper-virulent isolate of the pathogen, in the factorial experiment with a completely randomized design with four replications for each treatment. Petri plates were maintained at 25±1°C for five days. Then diameter of pathogen's colony was determined in different concentration of extracts and control and the inhibition percent of growth in pathogen colony was calculated by following standard formula. Data were subjected to ANOVA by SAS 9.2 software and treatment means were compared with DMRT.

Nano-particles synthesis from colony's extract of the premium isolate of T. harzianum: Colony's extract of the premium isolate of *T. harzianum* was mixed by Shelar and Chavan (2015) method with different concentrations of silver-nitrate solution and the optimal density of silver-nitrate was determined at a wavelength of 500-400 nm by observing the color change of the solution and by measuring its absorb value with Visible Spectrophotometer-Violet-Ultra. The produced solution was condensed and dried by Veerasamy *et al.* (2011) method. Then formation of nanoparticles was evaluated by dynamic light scattering [DLS: Malvern company, model: Nano S (Red badge), model number: ZEN1600] by Khlebtsov and Khlebtsov (2011) method, and scanning electron microscopy (SEM: Phenom ProX) by Lim *et al* (2005) method.

The impact of nano-synthesized product on the pathogen in-vitro: 100, 300, 500, 700, 900, 1000, 1100, 1300, 1500, 1700, 1900 and 2000 ppm concentrations of nano-synthesized product in PDA medium were tested in the inhibition of colony growth of hyper-virulent isolate of the pathogen by Kim *et al.* (2012) method and the inhibition's percent of pathogen's colony growth were calculated by following standard formula. The EC50 concentration of the nano-product was defined for the pathogen of analyzing data with SPSS16 software.

Testing the impact of nano-synthesized product on the disease severity in-vivo: Root of 21-days tomato seedlings of two varieties, i.e. Rio-grand and Falat (a native cultivar), was treated with concentration of EC_{50}, EC_{20}, EC_{80} of nano-product and optimum density solution of silver-nitrate individually for 30 minutes. Control seedlings were treated with sterilized distilled water. Treated seedlings were transferred to plastic pots with a diameter of 15cm and height of 20cm of topsoil, sterilized fertilizer and sand (1:1:1). Three plants were examined in each pot and six pots were considered for each treatment. Five milliliters of suspension of 10^7 spores of the pathogen in each milliliter of distilled water, were poured on the soil, after repeated hits of a sterilized nail to the soil around the root of each seedling, 24 hr after the root treatment. Pots were kept in the greenhouse and were irrigated every three days. Factorial experiment plan with completely randomized statistical design was followed. Treatments of the experiment were for two tomato varieties containing different concentration of nano-product, silver-nitrate, health and disease control. After two months, indicators of disease severity and length of plants' stem were recorded. Data were submitted to ANOVA by SAS 9.2 software and treatment means were compared with DMRT.

RESULTS AND DISCUSSION

Identifying the pathogen and pathogenicity test of their isolates: Two isolates of pathogen were identified based on the disease symptoms, characteristics of the colony and spores, as *Fusarium oxysporum* f. sp. *lycopersici*. This soil-borne pathogen also is common in central and southern parts of Iran, and most countries in fields and tomato-growing greenhouses (Reis *et al.* 2005, Ershad 2009, Amini *et al.* 2014, Amutha and Darwin Christdhas 2017). Two treated tomato varieties were inoculated with these isolates, showed symptoms of the disease after two months. Most of the disease severity arose in the Rio-Grande variety with isolate one of the pathogen.

Impact of colony's extract of T. harzianum isolates on colony growth of the pathogen in-vitro: Colony's extract of all *T. harzianum* isolates significantly reduced the colony growth of the pathogen *in vitro*, but the impact of *T. harzianum* 39 extract was more in all concentrations than other isolates extracts and was identified as the premium isolate (Fig 1). Colony's extract of two isolates of *T. harzianum* also, have inhibited colony growth of an important phytopathogenic fungus, *Rhizoctonia solani* Kuhn (Vinale *et al.* 2006). Some species of *Trichoderma* cause the destruction of hyphal wall and death of many plants pathogenic fungi with secreting enzymes such as chitinase, beta-1,3-glucanase, beta-1,6-glucanase, alpha-1,3-glucanase and proteases. They also produce the secondary metabolites such as 6-pentyl-a-pyrone (6PP), koningic acid, heptelidic acid, viridin, terpenes, alpha-pyrones, polyketides,

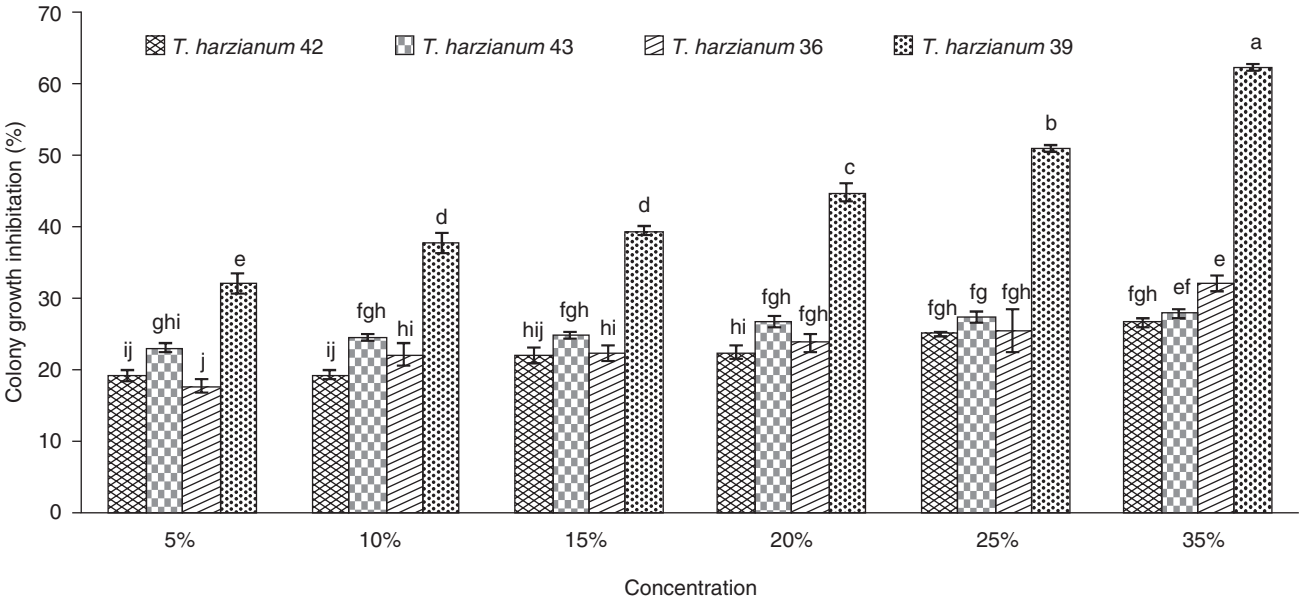

Fig 1 The impact of different colony's extract concentrations of *Trichoderma harzianum* isolates on the colony growth of *Fusarium oxysporum* f. sp. *lycopersici.*

which have increasing effect in combination with cell wall degrading enzymes (Vinale *et al.* 2006).

Nano-synthesized product of colony's extract of the premium isolate of T. harzianum and its impact on colony growth of the pathogen in-vitro: The color of the mixture containing *T. harzianum* 39 extract and silver ions was changed from pale-yellow to brown after 24 h, which indicates the formation of nanoparticles from the fungal extract. The light absorption peak at a wavelength of 400-600 nm, also confirmed the presence of nanoparticles in the solution. Absorption value was increased by increasing the concentration of silver-nitrate. Since nanoparticles have the maximum absorption at a wavelength of 400-500 nm, the density of 18 mmol of silver-nitrate with the maximum absorption at a wavelength of 472 nm, was known as optimum concentration and was selected for doing other steps of the research. DLS diagram and the figure of SEM confirmed the presence of nano-particles with 50-65.7 nm diameter.

Silver-nitrate caused the conversion of premium isolated extract of *T. harzianum* 39 to nanoparticles bio-fungicide. Colony's extract of some *Trichoderma* species also have been converted to nano-products with this method (Devi *et al.* 2013, Shelar and Chavan 2015). The antimicrobial effect of nanoparticles depends on their size and whatever nanoparticles be smaller, have more contact surface with microbial cells and their influence is further on microbial cells and thus their antimicrobial activity is additional (Rai *et al.* 2009). Nanoparticle produced from the extraction of some *Aspergillus foetidus* isolates also had been with a diameter of 78nm (Roy *et al.* 2013). Nanoparticles product with different concentration reduced the growth of the pathogen colony *in-vitro*, with EC_{50} 441.9 ppm.

Impact of nano-synthesized product on disease severity in-vivo: The result of analysis of variance of the experimental data showed that the nano-product with different concentration caused a significant reduction of the disease severity and their effects were more than the silver-nitrate (Table 1).

Table 1 The impact of different concentrations of nano-synthesized product from *Trichoderma harzianum* 39 colony's extract and silver-nitrate on the tomato *Fusarium* wilt disease severity in greenhouse*.

Vascular browning (cm)	Stem height (cm)	Disease severity index	Treatment	Tomato variety
9.59 a	38.94 c	4 a	Check (diseased)	Falat
7.61 abc	39.20 c	3.5 abc	$AgNO_3$ (18 mmol)	
6.34 bc	43.67 abc	2.83 cd	EC_{20}	
4.83 c	43.59 abc	2.5 cd	EC_{50}	
3.62 c	49.61 ab	2.17 d	EC_{80}	
0 d	52.04 a	1 e	Check (healthy)	
8.41 ab	36.20 c	3.66 ab	Check (diseased)	Rio-grande
6.54 bc	41.07 bc	3.33 abc	AgNO3 (18 mmol)	
4.55 c	45.08 abc	2.5 cd	EC_{20}	
3.31 cd	50.40 ab	2.17 d	EC_{50}	
2.44 cd	49.94 ab	2 d	EC_{80}	
0 d	51.17 a	1 e	Check (healthy)	

*Numbers with same letter have not significant differences at $P \leq 0.01$ (Duncan's multiple range test =DMRT).

Nano-product had the highest impact on disease severity in both varieties of tomato in two concentrations of EC_{50} and EC_{80}, at 1% statistical level. They did not have a significant difference with healthy control in the average stem length and significantly decreased height of vascular browning more than the silver-nitrate (Fig 2). So the concentration equal to EC_{50}=441.9 ppm of this nano-product is the most effective density against the disease with regard to the economy.

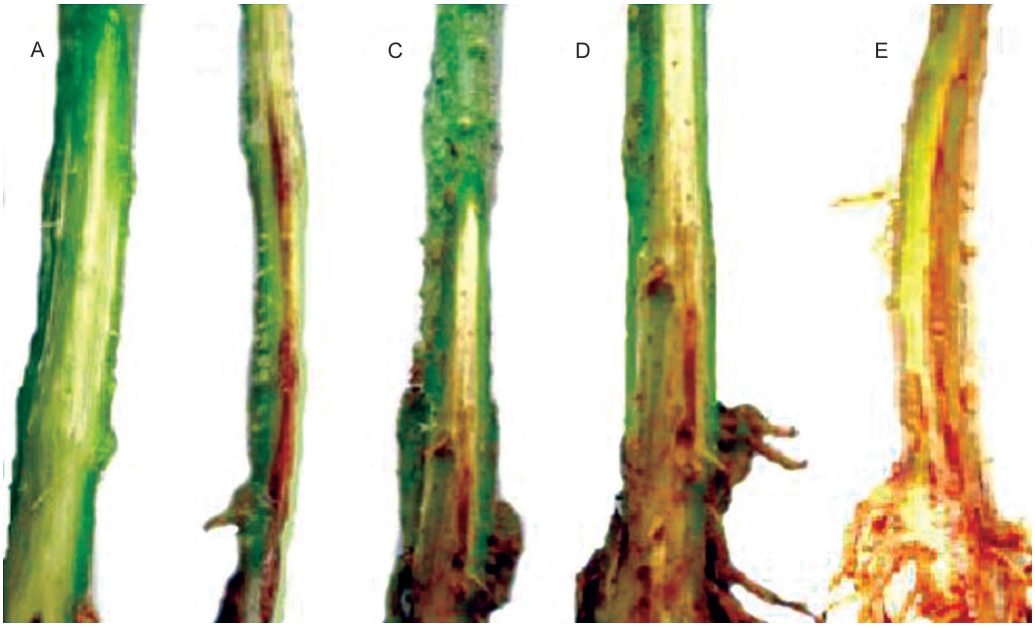

Fig 2 Vascular browning caused by *Fusarium oxysporum* f. sp. *lycopersici* in tomato treated by: A. Healthy check, B. Diseased check, C. EC_{80} concentration of nano-synthesized product from *Trichoderma harzianum* 39 colony extract, D. EC_{50} concentration of nano-synthesized product, E. 18 mmol of $AgNO_3$

These results showed that the nano-extract of *T. harzianum* 39 has more effective substances than silver-nitrate. *Trichoderma* species, specially *T. harzianum*, produce many antifungal metabolites such as. anthraquinones (e.g. trichodermaol, pachybasin, chrysophanol and emodin), daucane, simple pyrones, koininins, viridins, viridiofungins and trichodenones, those chemical structure is well known and described (Reino *et al.* 2008)

Fusarium wilt is a major disease of tomato in the world. Biological control of the disease is a safe and secure method for our environment. This study showed that the colony's extract of four isolates of *T. harzianum* have significant ability to inhibit the colony growth of *Fusarium oxysporum* f. sp. *lycopersici*. Synthesized nanoparticles had 50-65.7nm diameters. Synthesized nanoparticles product with EC_{50} 441.9 ppm had inhibitory effect of the pathogen colony growth *in vitro*. This nano-product also caused significant reduction of disease severity in greenhouse condition and its effect was more than silver-nitrate alone. Therefore, its use can be further evaluated large area for disease management. Potential of *T. harzianum* nano-extract for biological control of the disease is being reported for the first time as per our knowledge.

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