Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Evaluation of the efficacy of different concentrations of nano-capsules containing *Talaromyces flavus* with two forms of powder and suspension in reducing the incidence of cotton Verticillium wilt

Avaliação da eficácia de diferentes concentrações de nanocápsulas contendo *Talaromyces flavus* com duas formas de pó e suspensão na redução da incidência de murcha de Verticillium do algodoeiro

M. H. Shabani[a], L. Naraghi[b*], M. Maleki[a] and M. Negahban[c]

[a]Islamic Azad University, College of Agriculture, Department of Plant Pathology, Varamin, Iran

[b]Department of Plant Diseases Research, Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

[c]Department of Pesticides Research, Iranian Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

Abstract

Previous domestic and foreign studies have shown the significant effect of *Talaromyces flavus* on growth inhibition of some important plant pathogens including *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *cucumerinum*. In Iran, it is necessary to produce new formulations of this fungus based on modern technologies given the importance of attracting companies producing biological control agents and transferring the technical knowledge of mass production of formulations of these agents to them. In the present study, based on the method presented in the Pesticide Research Department of the Iranian Plant Protection Research Institute, two types of *T. flavus* formulations in the form of nano-capsules containing *Talaromyces flavus* with two forms of powder and suspension were prepared using nanotechnology. In the next step, during the greenhouse examination, the efficiency of each of these new formulations in concentrations of one to five per thousand for soil addition method and concentration of five per thousand for seed impregnation method (six treatments for each of the two new formulations) was compared with the registered formulation of Talaromin in two methods of seed impregnation and soil addition with healthy control and infected control to control cotton Verticillium wilt disease, in the form of a randomized complete block design with 16 treatments and 5 replications. After statistical analysis of the data obtained by Duncan's Multiple Range Test by MS TAT C software, the results showed that in terms of disease severity among treatments with the previous formulation (Talaromin) with each of the methods of soil addition and seed impregnation, there was no statistically significant difference between nano-suspension with each of the concentrations of one, four and five per thousand by the soil addition method and nano-powder with each of the concentrations of two and three per thousand by soil addition method, and the mentioned treatments were included in one statistical group in terms of disease severity with healthy control.

Keywords: nano-formulation, *Talaromyces flavus*, biological control, plant diseases.

Resumo

Estudos anteriores nacionais e internacionais mostraram o efeito significativo de *Talaromyces flavus* na inibição do crescimento de alguns importantes patógenos de plantas, incluindo *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *lycopersici* e *Fusarium oxysporum* f. sp. *cucumerinum*. No Irã, é necessário produzir novas formulações desse fungo com base em tecnologias modernas, dada a importância de atrair empresas produtoras de agentes de controle biológico e transferir para elas o conhecimento técnico de produção em massa das formulações desses agentes. No presente estudo, com base no método apresentado no Departamento de Pesquisa de Pesticidas, do Instituto Iraniano de Pesquisa em Proteção de Plantas, dois tipos de formulações de *T. flavus*, na forma de nanocápsulas contendo *T. flavus* com duas formas de pó e suspensão, foram preparados usando nanotecnologia. Na etapa seguinte, durante o exame em casa de vegetação, a eficiência de cada uma dessas novas formulações em concentrações de um a cinco por mil para o método de adição de solo e de cinco por mil para o método de impregnação de sementes (seis tratamentos para cada uma das duas novas formulações) foi comparada com a formulação registrada de Talaromin em dois métodos de impregnação de sementes e adição de solo com controle sadio e controle infectado para

*e-mail: sheredko.s@yahoo.com
Received: March 27, 2022 – Accepted: May 29, 2022

controle da murcha de Verticillium do algodoeiro, na forma de delineamento em blocos completos casualizados com 16 tratamentos e 5 repetições. Após análise estatística dos dados obtidos pelo Duncan's Multiple Range Test por meio do software MS TAT C, os resultados mostraram que, em termos de severidade da doença entre os tratamentos com a formulação anterior (Talaromin), com cada um dos métodos de adição de solo e impregnação de sementes, não houve diferença estatisticamente significativa entre a nanossuspensão com cada uma das concentrações de um, quatro e cinco por mil pelo método de adição de solo e entre o nanopó com cada uma das concentrações de dois e três por mil pelo método de adição de solo, e os tratamentos mencionados foram incluídos em um grupo estatístico em termos de gravidade da doença com controle saudável.

Palavras-chave: nanoformulação, *Talaromyces flavus*, controle biológico, doenças de plantas.

Introduction

In studies conducted in Iran, the favorable results of *Talaromyces flavus* antagonist fungus on controlling some important soil pathogens such as *Verticillium dahliae*, *Verticillium albo-atrum*, *Fusarium oxysporum* and *Rhizoctonia solani* in several crops including cotton, sugar beet, potatoes, tomatoes and greenhouse cucumbers have been proven (Whipps, 2001; Naraghi et al., 2010a; Naraghi et al., 2010b; Naraghi et al., 2010c; Naraghi et al., 2012a). Also, application of this fungus in the fields in the propagated form in solid fermentation on plant residues or their mixture with peat soil reduced the incidence of disease and increased yield in the above-mentioned crops. In cotton, 50% decrease in the percentage of Verticillium wilt disease, 37% decrease in seedling death and 30% increase in yield were observed. In potato, 40% decrease in disease percentage and 17% increase in yield were observed (Naraghi et al., 2014b). In sugar beet, 93% increase in the number of healthy seedlings and 50% increase in yield (Naraghi et al., 2014a) were observed. In tomato, 27% decrease in disease severity and 23% increase in yield (Niya et al., 2015) were observed. In greenhouse cucumber, 30% decrease in disease severity and 7% increase in yield were observed (Naraghi et al., 2017).

Since the issue of marketing and attracting relevant consumers is important in mass production and commercialization of biological agents, (Husen et al., 2007; Alimi et al., 2006; Kaewchai et al., 2009; Pereira et al., 2009; Amatuzzi et al., 2018; Oliveira et al., 2021), the issue of commercialization of the biological agent *T. flavus* and producing its various bio-formulations, including nano-formulations seems to be necessary. In recent decades, nanotechnology has expanded significantly in various chemical, pharmaceutical, medical and chemical pesticides areas. An issue that requires research and development in the area of nano-pesticides is the phenomenon of pest resistance to pesticides. Thus, the introduction of nano-pesticides to researchers will lead to a boom in research and development in this relatively new area. Due to environmental problems and costs due to the use of large amounts of conventional pesticides as well as problems due to pest resistance to these pesticides, research and development in the area of nano pesticides can be considered as a necessity. The use of biodegradable polymers in the production of high-performance nano-emulsions and nano-capsules made from natural and biodegradable materials can be an effective step in this regard. To increase efficiency and reduce environmental hazards, encapsulation formulations seem to be the best option (Maji et al., 2014).

Hence, production of bio-formulations in nano and micro forms creates a controlled ability, increases strength and stability and protects the active substance in adverse environmental conditions such as light and moisture. Also, application of nano-encapsulated formulations helps to reduce the pesticide dose and cost saving and protect the environment and reduce its environmental risks and increase the export of the product (Martín et al., 2010). Nanoparticles have a larger surface area than micro-particles, which increases their active surface and their controlled release. Also, another advantage of particle nanometers is that they do not stimulate the immune system of humans and animals and are rapidly eliminated from the body (Guan et al., 2008).

Nano-capsule technology, which contains fungicide or pesticide molecules at nanoscale, is one of the methods to produce a pesticide formulation, making pest removal easier and faster (Guan et al., 2008). An emulsion is a heterogeneous system consisting of two immiscible liquids, one of which is dispersed in the other in droplets. Emulsions with droplet sizes of about nanometers and in the range of 20 to 200 nm are called nano-emulsions (Ostertag et al., 2012). The unique structure and properties of nano-emulsions compared to conventional emulsions have created advantages for their application in many industries. Applications of nano-emulsion systems in industry include their role in removing the coating and controlling the release of beneficial compounds such as essential oils, vitamins, etc. (Kah and Hofmann, 2014).

A tendency to make nanoparticles that are more degradable in the environment and have high efficiency has recently received much attention. Thus, the use of biodegradable polymers in the production of high-performance nano-emulsions and nano-capsules made from natural and biodegradable materials such as essential oils of medicinal plants can be an effective step in this area. Application, production and environmental considerations of nano-pesticides show that research and development can be effective in reducing the phenomenon of pest resistance to pesticides (Ebrahimnejad et al., 2011). The nano-formulation prepared by microorganisms such as bacteria and fungi is used in biological control against insects and facilitates the entry of agents into the body of insects (Salunkhe et al., 2011). Pirimphos nano-insecticide formulation has made this formulation be kept well in the dark and remain more stable (Wan-Jun et al., 2010).

Nano-formulation extracted from plants against common pesticide-resistant pests increased pesticide effect by up to five times (Rajakumar and Rahuman, 2011). Nano-permethrin formulation by evaporation of

oil solvent in water could show much better effects in controlling Culex mosquito larvae (Anjali et al., 2010). The lethal properties of Bacillus thuringiensis have been examined using chitosan polymer nanoparticles on Anopheles mosquito larvae (Zhang et al., 2015). In addition, by using dextrose and gelatin polymers, bio-pesticides containing Beauveria, Metharhisium and Paecilomyces against Hypothenemus hampei were prepared (Niranjana, 2004). Beauveria bassiana, a formulation containing silver nanoparticles, was also used as larvicide (Prabakaran et al., 2016). In a research on the disinfecting effects of barley and sunflower seeds with silver nanoparticles containing fungicide on mycorrhizal coexistence, results showed that in the treatment of seeds with nano-fungicide compared to conventional fungicide, the absorption of minerals by the root and consequently vegetative traits increased significantly. Coating materials for encapsulation include gum, starch, gelatin and polymers. Chitosan and phospholipids have also been used recently. Based on recent studies, it seems that the use of nano-capsulation techniques and their components in the control of storage pests can play a major role in increasing their efficiency and durability (Prasad et al., 2014).

Many plant compounds that have insecticidal properties are highly evaporated and sensitive to decomposition. Loading of plant compounds in nanoparticles leads to controlled release and delayed decomposition and evaporation. There are limited articles on the insecticidal effect of nano-emulsions and nano-capsules loaded with compounds with pesticidal properties against pests (Ishaaya et al., 2013). There are several methods for producing nanoparticles or nano-capsules, for example, the polymerization method, which is one of the fastest methods in which aqueous continuous phase is mixed with the organic continuous phase (Kirthi et al., 2011). Also, one of these methods is coagulation and ion gel formation using biodegradable hydrophilic polymers such as chitosan, gelatin, sodium alginate, which is actually a mixture of two aqueous phases in a polymer phase such as chitosan and sodium alginate. In this method, positive charge in polymer is integrated with negative charge with crosslinks such as sodium triphosphate or calcium chloride and form capsules (Ebrahimnejad et al., 2011).

Two reports in the medical and agricultural areas have been presented so far for the preparation of nanoparticles from the fungus studied in the present study (Penicillium) (Priyadarshini et al., 2014; Khan and Jameel, 2016). In the medical area, the live fungus Penicillium fellutanum was used to prepare nanoparticles against the pathogenic fungus Candida albicans, and in the agricultural area, the extract of the fungus *Talaromyces flavus* (teleomorph of the fungus Penicillium dangeardii) was used to prepare the nanoparticles against some plant pathogens (Priyadarshini et al., 2014; Khan and Jameel, 2016). In the last twenty years, there have been significant reports on the preparation of bio-formulations containing antagonistic fungi using solid and liquid fermentation and their optimization at different stages of construction (Pascual et al., 1999; Budge and Whipps, 2001; Schuster and Schmoll, 2010; Damaso et al., 2012; Kakvan et al., 2013; Sargin et al., 2013). For example, Pascual et al. (1999) prepared a solid bio-formulation affected by Epicoccum nigrum on wheat. After examining alcoholic solutions including glycerol, mannitol and arabitol on sporulation of this fungus, they reported the most significant increase in sporulation by glycerol. In addition, Sargin et al. (2013) compared different drying methods of this bio-formulation to increase the efficiency of bio-formulation containing Trichoderma harzianum EGE-K38. Studies have indicated that the use of compounds containing mineral elements including manganese, iron, zinc and phosphorus in production of biological formulations containing antagonistic fungi has increased their stability (Vasane and Kothari, 2008; Lee and Lee, 2009).

Method

Bio-formulations such as Ketomium containing *Chaetomium globosum* and *Ch. Cupreum* containing *T. harzianum* and *T. viride*, Soil Gard containing *Gliocladium virens*, Trichodex containing *T. harzianum*, *Pisolithus tinctonus* and *Glomus intraradices*, Trichodermin containing *T. harzianum* and Protus WG containing *Talaromyces flavus* have been commercially registered outside of Iran so far (Shabgah et al., 2021; Koch, 1999; Kaewchai et al., 2009). In Iranian research, the use of Iranian bio-formulation called H-Trichomix has been reported. Also, in Iran, the research history for making bio-formulations containing different *T. flavus* isolates has been as follows:

T. flavus antagonist fungus was isolated and identified for the first time in Iran from the soil around the roots of cotton plant located in a field in a research station of Golestan province. The results of laboratory studies on the effects of inhibitory mechanisms of different *T. flavus* isolates on the growth of some soil pathogens in several crops showed that among the studied mechanisms including mycoparasitism, production of volatile and non-volatile compounds, and production of volatile compounds has the highest level of growth inhibition of the Verticillium wilt agent (*V. dahliae*). In another research, it was revealed that common antagonistic mechanisms among *T. flavus* in three crops of potato, tomato and greenhouse cucumber were mycoparasitism and production of volatile compounds for *V. albo-atrum*, mycoparasitism for *F. oxysporum* and production of non-volatile compounds for *R. solani*. In other studies, to use the mentioned fungus in greenhouse and field, bio-formulation containing it was prepared using solid substrates including fertilizer grown with earthworm, wheat straw, wheat bran, rice bran, wheat straw combined with wheat bran, perlite mixed with sugar supplement and peat soil mixed with sugar supplement and among them, the best substrates in terms of efficiency in increasing sporulation and stability for *T. flavus* isolates were related to cotton and potato, and rice bran and for *T. flavus* isolates, they were related to tomatoes and greenhouse cucumbers, peat soil mixed with sugar supplement.

The use of rice bran substrate for *T. flavus* propagation has also been reported in research conducted on the biological control of garlic white rot and the enhancement of its vegetative traits using the fungus. In addition, for biological control of seedling death and root rot

in cotton and cucumber, this substrate has been used for the multiplication of antagonistic bacteria such as *Pseudomonas fluorescens* (Ardekani et al., 2010; Khabbaz and Abbasi, 2014; Mehdizadehnaraghi et al., 2015). The results of greenhouse experiments in the area of biological control with Verticillium wilt disease of potato, tomato and greenhouse cucumber caused by *V. albo-atrum* by bio-formulations containing different isolates of *T. flavus* showed that these isolates were significantly effective in decreasing the disease index and increasing vegetative traits such as root length, crown length, height, fresh weight and dry weight of the above plants (Naraghi et al., 2010a, b, c; Naraghi et al., 2012b, c). Also, based on the results obtained from laboratory and greenhouse studies in the area of controlling Fusarium wilt factors of greenhouse cucumber and tomato by bio-formulations containing different isolates of *T. flavus*, in addition to determining the most effective bio-formulation, the method of using each of them was also determined in tomato fields and cucumber greenhouses. Also, field studies were conducted on the possibility of biological control of Verticillium wilt disease and cotton seedling death, sugar beet seedling death, Verticillium wilt of potato and Fusarium wilt of tomato and cucumber using *T. flavus* bio-formulation. The results showed that the application of this bio-formulation in addition to significantly reducing the disease index also caused a significant increase in yield (Naraghi et al., 2014a, b; Niya et al., 2015).

Results and Discussion

Experimental details

Production of nano-capsulated bio-formulation containing biocontrol fungus Talaromyces flavus in suspension form

The production of nano-capsule involves a combination of polymerization and crosslinking methods. It occurred by making changes in accordance with the growth conditions of biocontrol fungi (change in the amount or type of polymer, surfactants and oils, fatty acids and the amount of stirrer, temperature). In the polymerization process, the organic phase consisted of castor vegetable oil with a mixture of biological fungi, which was added to the aqueous phase consisting of hydrophilic polymer monomers such as a mixture of polymer alginate, starch and chitosan. Then, calcium chloride cross-linker as well as surfactants and associated materials, and fatty acid oils were added to two phases, and homogenized at 35 ° C at 5000 to 10,000 rpm. Finally, polymer particles were formed as capsules around the particles of biological fungi.

Production of nano-capsulated bio-formulation containing biocontrol fungus Talaromyces flavus in powder form

In powder nano-formulation, the suspension containing the spores of biological fungi in the aqueous phase was expanded to include maltodextrine, xanthan gum, fatty acid, ethanol amid, and oleic acid and after being exposed to homogenizer for three hours at 12000 rpm at 25 ° C, it was completely powdered. The properties of the nano-capsulated bio-formulation and its sizing are presented in Table 1 and Figure 1 respectively.

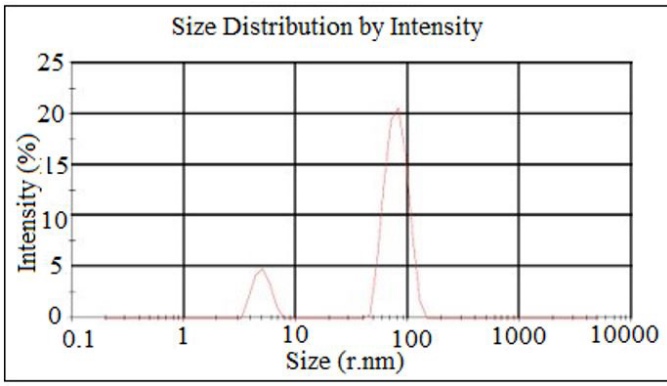
Preparation of T. flavus bio-formulation based on previous technical knowledge (Talaromin)

To prepare a solid formulation, an effective T. flavus isolate in the collection of fungi of the laboratory of beneficial microorganism's research and modified method of Naraghi et al. (2010a) were used. Accordingly, some rice bran was soaked in water (30-35 ° C) for 24 hours, and then, it was spread on large filter paper and was dried. In the next step, 250 g of rice bran was sterilized in cellophane bags in an autoclave (1.5 atm, 121 ° C for 20 minutes). In the next step, to prepare the bio-formulation, a suspension containing 40 ml of sterile distilled water and six half-cm pieces of 10-day culture medium of T. flavus isolate was poured into cellophane bags (Figure 2).

Then, one of the stabilizing compounds such as dicycloserine or sodium nitrate was added to the culture media (10 ml of the supplement solution at the rate of 20 g / l for 250 g of each medium) based on the amount of adding supplements (Heidary et al., 2017). For the growth of T. flavus isolate, the cellophane bag was incubated at 30 ° C for one and a half to two months. During this time, 20 ml of sterile distilled water was added to moisten if dryness was observed. After this period, the contents of the cellophane bag were spread on filter paper for drying, and it was used as a bio-formulation based on previous technical knowledge.

Table 1. Properties of the nano-capsulated bio-formulation.

Temperature	**25**	**Material RI (refractive index)**	**1.54**
Count rate	325.7	Dispersant RI	1.33
Cell Description	glass cuvette with square aperture	Viscosity	0.887
Duration Used	60	Z-average (r.nm)	87.12
Measurement position	4.65	PDI (polydispersity index)	0.257
Attenuator	10	Intercept	0.943



	Size (r.nm)	Intensity (%)	Width (r.nm)
Peak 1	80.39	84.5	17.37
Peak 2	5.005	15.5	0.8315
Peak 3	0.000	0.0	0.000

Figure 1. Size distribution of nano-capsul.

Figure 2. An overview of the steps of preparing *Talaromyces flavus* bio-formulation based on previous technical knowledge.

Preparation pathogenic inoculum of cotton Verticillium wilt and determination of its consumption for greenhouse use

In this step, VD-Co-P-G-22 isolate (isolate of Verticillium dahliae obtained from the stem of cotton plant in Gorgan field) is used to prepare the pathogenic inoculum of Verticillium wilt. The pathogenicity of this isolate has been proven in previous studies on cotton. To prepare the pathogenic inoculum for Verticillium wilt, modified method of Cimen and Basaran (2016) was used. First, V. dahliae isolate was cultured on Potato Dextrose Agar (PDA) medium and stored at 22 ± 1 ° C. After ten days and observing the microsclerotia on the surface of the petri dish, fungus was cultured on the wheat substrate for 10 days. For this purpose, 200 g of washed wheat seeds with 50 ml of distilled water were transferred into each cellophane bag and after sterilization, it was autoclaved at 120 °C and 1 atm pressure for 15 minutes, fungus suspension was applied on it. Then, 40 ml of fungus suspension was applied on it. To prepare the fungus suspension, six 5-mm pieces of ten-day fungus culture were transferred into 40 ml of sterile distilled water. The cellophane bags were stored in a 25 °C incubator for three weeks. Then, the contents of the cellophane bags were dried on large filter paper, and were used as a pathogenic inoculum in the greenhouse.

To determine the level of consumption of Verticillium wilt pathogenic inoculum in the greenhouse based on 200 microsclerot per gram of potting soil (Naraghi et al., 2012c), the required amount of consumed inoculum was estimated.

Accordingly, by calculating the number of microsclerotes per gram of inoculum, the amount of inoculum needed for the used soil used in each pot was determined.

To determine the number of microsclerots per gram of inoculum, a suspension of inoculum with a dilution of 0.001 was prepared and the number of microsclerot in 1 ml was counted by 40% magnification of the Olypmus BX40 LED Microscope. By multiplying the number obtained in the dilution coefficient photo, the number of microsclerot in 1 g of fertilizer was determined and accordingly, the amount of fertilizer used for the used soil in the pot was determined.

Evaluating the efficacy of T. flavus nano-formulations in the control of cotton Verticillium wilt diseases

To evaluate the efficacy of *T. flavus* nano-formulations in controlling the studied diseases (cotton verticellium wilt and Fusarium wilt), two separate experiments were performed for each of the mentioned diseases. Each experiment was performed in a randomized complete block design with 16 treatments and 4 replications. The treatments included as follows: Treatments 1 to 5: *T. flavus* nano-capsule in suspension with concentrations of 1 to 5 per thousand in soil addition method. Treatment 6: *T. flavus* nano-capsule in suspension by seed impregnation method. Treatments 7 to 11: T. flavus nano-capsules in powder form with concentrations of 1 to 5 per thousand in soil addition method. Treatment 12: *T. flavus* nano-capsule in powder form by seed impregnation method. Treatment 13: registered biological fungicide talaromyces flavus by soil addition method. Treatment 14: registered biological fungicide *Talaromyces flavus* by seed impregnation method. Treatment 15: healthy control. Treatment 16: infected control. In these studies, susceptible cultivar of Varamin was used for cotton plants.

Evaluation of Verticillium wilt disease by determining the severity of the disease according to Hao et al. (2005) starts from one month after implantation and will continue for up to four months (Wokoma, 2008). Finally, the comparisons among the treatments were performed by analyzing the data by Duncan's Multiple Range Test using MS TAT C software. To determine the severity of the disease, the incidence of the disease was first determined by observing its symptoms using a scale consisting of six grades (Liu et al., 1995) as follows:

0 = no symptoms
1= Leaf chlorosis and plant wilt less than 25%
2=Leaf chlorosis and plant wilt from 25 to 50%
3=Leaf chlorosis and plant wilt from 51 to 75%
4=Leaf chlorosis and plant wilt from 76 to 100%
5=The plant is dead or completely destroyed

Then, the disease severity percentage for each treatment was calculated according to the formula mentioned for the pathogenicity test as follows (Equation 1):

$$infection\ sevetiry\ percentage = \frac{\sum (n_i \times v_i) \times 100}{N \times V} \quad (1)$$

n: number of plants per grade; v: number of each grade; N: total number of plants; V: Number of highest grade of infection (5).

In the soil addition method, the level application of each of the new formulations for the surface of the tested pots was calculated based on the application of liquid biological fertilizers with a concentration of one to six per thousand and increasing the required amount of each concentration with 400 liters of water for one hectare of field (Seefeldt et al., 2001).

Determining the efficacy of T. flavus bio-formulation based on previous technical knowledge and different concentrations of nano-capsules containing T. flavus in the control of cotton Verticillium wilt disease

The test to determine the efficacy of T. flavus bio-formulation based on previous technical knowledge and different concentrations of nano-capsules containing T. flavus in controlling Verticillium wilt disease of cotton was significant at the probability level of 1%. According to the results of grouping the mean percentage of disease severity in different treatments, all treatments were divided into three statistical groups (Table 1). All treatments containing each of the nano-suspension and nano-powder formulations in the method of adding to the soil with different concentrations and the method of seeding and Talaromin fungicide with two methods of adding to the soil and seed impregnation compared to the infected control have a significant reduction in disease severity (Table 1). In this regard, seven treatments had a disease severity of 0 and were placed in a statistical group of healthy control (Table 2). These treatments were: Talaromin by soil addition method, Talaromin by seed impregnation method, nano-suspension by soil addition method with concentrations of one, four and five per thousand and nano-powder by soil addition method with concentrations of two and three per thousand (Table 2).

The general results of this study showed that both new types of nano-capsule formulations containing *T. flavus* with two forms of suspension and powder are effective in reducing the incidence of the disease. The results of greenhouse studies showed that all nano-formulations significantly reduced the incidence of the studied disease compared to the infected control. It suggests that under greenhouse conditions, there was more time to produce metabolites and it has been an important factor for the production of secondary metabolites of *T. flavus* (Zabihi et al., 2011; Petersen et al., 2019). The results of this study revealed that nano-suspension was more effective at minimum (one per thousand) and maximum (four and five per thousand) concentrations, while nano-powder was more effective at moderate concentrations (two and three per thousand). In addition, among the nano-capsules tested, the most effective nano-capsule in controlling the studied cotton disease was nano-powder by seed impregnation method due to ease of application. No study has been conducted so far in the area of agriculture to investigate the effect of nano-fungicides containing live fungal biological agents on plant diseases. However, domestic and foreign research results have been reported

Table 2. Grouping the mean percentage of cotton Verticillium wilt disease severity in different treatments including nano-formulations containing T. flavus and Talaromin fungicide.

Treatment	Mean percentage of cotton Verticillium wilt disease severity
Nano-suspension containing T. flavus at a concentration of one per thousand by soil addition method	8.13ef*
Nano-suspension containing T. flavus at a concentration of two per thousand by soil addition method	23.19bcd
Nano-suspension containing T. flavus at a concentration of three per thousand by soil addition method	17.33bcde
Nano-suspension containing T. flavus at a concentration of four per thousand by soil addition method	10.91def
Nano-suspension containing T. flavus at a concentration of five per thousand by soil addition method	10.00ef
Nano-suspension containing T. flavus by seed impregnation method	26.13abc
Nano-powder containing T. flavus with a concentration of one per thousand by soil addition method	14.00cde
Nano-powder containing T. flavus with a concentration of two per thousand by soil addition method	12.00def
Nano-powder containing T. flavus with a concentration of three per thousand by soil addition method	0f
Nano-powder containing T. flavus with a concentration of four per thousand by soil addition method	15.60bcde
Nano-powder containing T. flavus with a concentration of five per thousand by soil addition method	28.00ab
Nano-powder containing T. flavus by seed impregnation method	20.00bcde
Talaromin by soil addition method	11.33def
Talaromin by seed impregnation method	0f
Healthy witness	0f
Infected control	53.36a

*There is no statistically significant difference between the means with similar statistical letters at the 1% probability level.

regarding the production of nano-fungicides containing non-living biological agents and their effectiveness in controlling plant diseases. A foreign research has referred to production of nano-fungicides using titanium dioxide nanoparticles in the form of nano-emulsions to control some plant diseases (Abd-Elsalam and Alghuthayni, 2015). In Iran, nano-fungicides have been prepared using organic compounds and its effect on tobacco collar rot has been investigated. The results of this study showed that the synthesized nano-fungicide was effective in controlling tobacco collar rot (Sajjadi, 2014).

Conclusion

Overall, the results of this greenhouse study showed that concentrations of one to five per thousand microcapsules of *T. flavus* with two forms of suspension and powder by adding soil method and concentrations of five per thousand microcapsules of *T. flavus* with two forms of suspension and powder with impregnated method Seed production has the ability to control Verticillium wilt disease. To confirm these results, additional research is needed at the fields.

Acknowledgements

The authors would like to thank the support of Iranian National Science Foundation (INSF) for financial support for the implementation of this research.

References

ABD-ELSALAM, K.A. and ALGHUTHAYNI, M.A., 2015. Nanobiofungicides: is it the next generation of fungicides? *Journal of Nanotechnology and Materials Science*, vol. 2, no. 2, pp. 38-40. http://dx.doi.org/10.15436/2377-1372.15.013.

ALIMI, T., AJEWOLE, O.C., OLUBODE-AWOSOLA, O.O. and IDOWU, E.O., 2006. Economic rationale of commercial organic fertilizer technology in vegetable production in Osun state of Nigeria. *Journal of Applied Horticulture*, vol. 8, no. 2, pp. 159-164. http://dx.doi.org/10.37855/jah.2006.v08i02.37.

AMATUZZI, R.F., CARDOSO, N., POLTRONIERI, A.S., POITEVIN, C.G., DALZOTO, P., ZAWADENEAK, M.A. and PIMENTEL, I.C., 2018. Potential of endophytic fungi as biocontrol agents of *Duponchelia fovealis* (Zeller) (*Lepidoptera crambidae*). *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 78, no. 3, pp. 429-435. PMid:29160362.

ANJALI, C.H., KHAN, S.S., GOSHEN, K.M., MAGDASSI, S., MUKHERJEE, A. and CHANDRASEKARAN, N., 2010. Formulation of waterdispersible nanopermethrin for larvicidal applications. *Ecotoxicology and Environmental Safety*, vol. 73, no. 8, pp. 1932-1936. http://dx.doi.org/10.1016/j.ecoenv.2010.08.039. PMid:20833431.

BUDGE, S.P. and WHIPPS, J.M., 2001. Potential for integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using Coniothyrium minituns and reduced fungicide application. *Phytopathology*, vol. 91, no. 2, pp. 221-227. http://dx.doi.org/10.1094/PHYTO.2001.91.2.221. PMid:18944397.

CIMEN, I. and BASARAN, M., 2016. Effect of solarization with fresh chicken manure on Verticillium wilt (*Verticillium dahliae* Klebb) and yield in eggplant. *African Journal of Biotechnology*, vol. 15, no. 52, pp. 2854-2863.

DAMASO, M.C.T., TERZI, S.C., FARIAS, A.X., OLIVEIRA, A.C.P., FRAGA, M.E. and COURI, S., 2012. Selection of cellulolytic fungi isolated from diverse substrates. *Brazilian Archives of Biology and Technology*, vol. 55, no. 4, pp. 513-520. http://dx.doi.org/10.1590/S1516-89132012000400005.

EBRAHIMNEJAD, P., DINARVAND, R., JAFARI, M.R., TABASI, S. and ATYABI, F., 2011. Characterization, blood profile and biodistribution properties of surface modified PLGA nanoparticles of SN-38. *International Journal of Pharmaceutics*, vol. 406, no. 1-2, pp. 122-127. http://dx.doi.org/10.1016/j.ijpharm.2010.12.022. PMid:21185365.

GUAN, H., CHI, D., YU, J. and LI, X., 2008. A novel photodegradable insecticide: preparation, characterization and properties evaluation of nano-Imidacloprid. *Pesticide Biochemistry and Physiology*, vol. 92, no. 2, pp. 83-91. http://dx.doi.org/10.1016/j.pestbp.2008.06.008.

HAO, Z., CHRISTIE, P., QIN, L., WANG, C. and LI, X., 2005. Control of Fusarium wilt of cucumber seedling by inoculation with an arbuscular mycorrhical fungus. *Journal of Plant Nutrition*, vol. 28, no. 11, pp. 1961-1974. http://dx.doi.org/10.1080/01904160500310997.

HEIDARY, S., IMANI, M. and MOSTAFAVI, S.M., 2017. A validated and rapid hplc method for quantification of human serum albumin in interferon beta-1a biopharmaceutical formulation. *MedBioTech Journal*, vol. 1, no. 1, pp. 26-30.

HUSEN, E.H., SIMANUNGKALIT, R.D.M., SARASWATI, R. and IRAWAN, I., 2007. Characterization and quality assessment of Indonesian commercial biofertilizer. *Indonesian Journal of Agricultural Science*, vol. 8, no. 1, pp. 31-38. http://dx.doi.org/10.21082/ijas.v8n1.2007.p31-38.

ISHAAYA, I., PALLI, S.R. and HOROWITZ, A.R., 2013. *Advanced technologies for managing insect pests*. Dordrecht: Springer.

KAEWCHAI, S., SOYTONG, K. and HYDE, K.D., 2009. Mycofungicides and fungal biofertilizers. *Fungal Diversity*, vol. 38, no. 1, pp. 25-50.

KAH, M. and HOFMANN, T., 2014. Nanopesticide research: current trends and future priorities. *Environment International*, vol. 63, no. 1, pp. 224-235. http://dx.doi.org/10.1016/j.envint.2013.11.015. PMid:24333990.

KAKVAN, N., HEYDARI, A., ZAMANIZADEH, H.R., REZAEE, S., and NARAGHI, L., 2013. Development of new bioformulations using Trichoderma and Talaromyces fungal antagonists for biological control of sugar beet damping-off disease. *Crop Protection*, vol. 53, no. 1, pp. 80-84.

KHAN, N.T. and JAMEEL, N., 2016. Antifungal activity of silver nanoparticles produced from fungus, *Penicillium fellutanum* at different pH. *Journal of Microbial & Biochemical Technology*, vol. 8, no. 5, pp. 440-443.

KIRTHI, A.V., RAHUMAN, A.A., RAJAKUMAR, G., MARIMUTHU, S., SANTHOSHKUMAR, T., JAYASEELAN, C. and VELAYUTHAM, K., 2011. Acaricidal, pediculocidal and larvicidal activity of synthesized ZnO nanoparticles using wet chemical route against blood feeding parasites. *Parasitology Research*, vol. 109, no. 2, pp. 461-472. http://dx.doi.org/10.1007/s00436-011-2277-8. PMid:21340566.

KOCH, E., 1999. Evaluation of commercial products for microbial control of soil-borne plant disease. *Crop Protection*, vol. 18, no. 2, pp. 119-125. http://dx.doi.org/10.1016/S0261-2194(98)00102-1.

LEE, S. and LEE, J., 2009. Color stabilization of low toxic antimicrobial polypropylene/poly (hexamethylene guanidine) phosphate blends by Taguchi technique. *Macromolecular Research*, vol. 17, pp. 411-416. http://dx.doi.org/10.1007/BF03218882.

LIU, L., KLOEPPER, J.W., and TUZUN, S., 1995. Induction of systemic resistance in cucumber against Fusarium wilt by plant growth promoting rhizobacteria.. *Phytopathology*, vol. 85, pp. 695-698

MAJI, R., DEY, N., SATAPATHY, B., MUKHERJEE, B. and MONDAL, S., 2014. Preparation and characterization of Tamoxifen citrate loaded nanoparticles for breast cancer therapy. *International Journal of Nanomedicine*, vol. 9, no. 11, pp. 3107-3118. PMid:25028549.

MARTÍN, Á., VARONA, S., NAVARRETE, A. and COCERO, M.J., 2010. Encapsulation and co-precipitation processes with supercritical fluids: applications with essential oils. *The Open Chemical Engineering Journal*, vol. 4, no. 1, pp. 31-41. http://dx.doi.org/10.2174/1874123101004010031.

NARAGHI, L., ARJMANDIAN, A., HEYDARI, A., SHARIFI, K. and AZAD, H.A., 2014a. A comparison between carbendazim fungicide and *Talaromyces flavus* in controlling Verticillium wilt of potato under field conditions. *International Journal of Agricultural Science and Research*, vol. 4, no. 1, pp. 89-100.

NARAGHI, L., HEYDARI, A., AZAD, H.A. and SHARIFI, K., 2012a. Antagonistic effects of *Talaromyces flavus* on some soil-borne pathogens of potato, tomato and greenhouse cucumber. In: *Proceedings of 20th Iranian Plant Protection Congress*, 25-28 August 2012, Shiraz, Iran. Tehran, Iran: Publihser Agricultural Research, Education and Extension Organization (AREEO), p. 278.

NARAGHI, L., HEYDARI, A., HESAN, A. and SHARIFI, K., 2014b. Evaluation of *Talaromyces flavus* and *Trichoderma harzianum* in biological control of sugar beet damping-off disease in the greenhouse and field conditions. *International Journal of Agricultural Science and Research*, vol. 4, no. 1, pp. 65-74.

NARAGHI, L., HEYDARI, A., REZAEE, S. and RAZAVI, M., 2012b. Biocontrol agent *Talaromyces flavus* stimulates the growth of cotton and potato. *Journal of Plant Growth Regulation*, vol. 31, pp. 471-477. http://dx.doi.org/10.1007/s00344-011-9256-2.

NARAGHI, L., HEYDARI, A., REZAEE, S., RAZAVI, M. and AFSHARI-AZAD, H., 2010a. Biological control of greenhouse cucumber Verticillium wilt disease by *Talaromyces flavus*. *Phytopathologia Mediterranea*, vol. 49, no. 1, pp. 321-329.

NARAGHI, L., HEYDARI, A., REZAEE, S., RAZAVI, M. and AFSHARI-AZAD, H., 2012c. Promotion of growth charasteristics in greenhouse cucumber and tomato by *Talaromyces flavus*. *International Journal of Agricultural Science and Research*, vol. 2, no. 3, pp. 129-141.

NARAGHI, L., HEYDARI, A., REZAEE, S., RAZAVI, M. and JAHANIFAR, H., 2010b. Study on antagonistic effects of *Talaromyces flavus* on *Verticillium albo-atrum*, the causal agent of potato wilt disease. *Crop Protection* vol. 29, no. 7, pp. 658-662. http://dx.doi.org/10.1016/j.cropro.2010.01.011.

NARAGHI, L., HEYDARI, A., REZAEE, S., RAZAVI, M., JAHANIFAR, H. and KHALEDI, E.M., 2010c. Biological control of tomato

Verticillium wilt disease by *Talaromyces flavus*. *Journal of Plant Protection Research*, vol. 50, no. 3, pp. 360-365. http://dx.doi.org/10.2478/v10045-010-0061-x.

NARAGHI, L., SHAHRIARY, D., SARPELEH, A., HEYDARI, A. and AFSHARI-AZAD, H., 2017. Decrease in incidence of cucumber Fusarium wilt in Varamin greenhouse using *Talaromyces flavus*. *International Journal of Agricultural Science and Research*, vol. 7, no. 4, pp. 143-154. http://dx.doi.org/10.24247/ijasraug201719.

NIRANJANA, S.R., 2004. Exploitation of entomopathogenic fungus *Beauveria bessiana* for efficient control of coffee berry borer in India. *Journal of Mycology and Plant Pathology*, vol. 34, no. 3, pp. 714-723.

NIYA, S.F., NARAGHI, L., OMMATI, F. and PIRNIA, M., 2015. Evaluation of the efficacy of the biological compound affected by *Talaromyces flavus* in controlling tomato Fusarium wilt disease in the field conditions. *International Journal of Agricultural Science and Research*, vol. 5, no. 2, pp. 153-164.

OLIVEIRA, J.C., SALES, J.F., RUBIO-NETO, A., SILVA, C.F., SOARES, M.A. and SILVA, F.G., 2021. Biological control in the germination of seeds from two species native of the Cerrado region. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 81, no. 1, pp. 105-113. http://dx.doi.org/10.1590/1519-6984.222279. PMid:32159616.

OSTERTAG, F., WEISS, J. and MCCLEMENTS, D.J., 2012. Low-energy formation of ediblenanoemulsions: factors influencing droplet size produced by emulsion phaseinversion. *Journal of Colloid and Interface Science*, vol. 388, no. 1, pp. 95-102. http://dx.doi.org/10.1016/j.jcis.2012.07.089. PMid:22981587.

PASCUAL, S., MELGAREJO, P. and MAGAN, N., 1999. Production of the fungal biocontrol agent Epicoccum nigrum by solid substrate fermentation: effect of water activity on accumulation of compatible solutes. *Mycopathologia*, vol. 146, pp. 83-89. http://dx.doi.org/10.1023/A:1007082829307.

PEREIRA, I., ORTEGA, R., BARRIENTOS, L., MOYA, M., REYES, G. and KRAMM, V., 2009. Development of a biofertilizer based on filamentous nitrogen- fixing cyanobacteria for rice crops in Chile. *Journal of Applied Phycology*, vol. 21, no. 1, pp. 135-144. http://dx.doi.org/10.1007/s10811-008-9342-4.

PRABAKARAN, K., RAGAVENDRAN, C. and NATARAJAN, D., 2016. Mycosynthesis of silver nanoparticles from Beauveria bassiana and its larvicidal, antibacterial, and cytotoxic effect on human cervical cancer (HeLa) cells. *RSC Advances*, vol. 6, no. 51, pp. 44972-44986. http://dx.doi.org/10.1039/C6RA08593H.

PRASAD, R., KUMAR, V. and PRASAD, K.S., 2014. Nanotechnology in sustainable agriculture: present concerns and future aspects. *African Journal of Biotechnology*, vol. 13, no. 6, pp. 705-713. http://dx.doi.org/10.5897/AJBX2013.13554.

PRIYADARSHINI, E., PRADHAN, N., SUKLA, L.B., PANDA, P.K. and MISHRA, B.K., 2014. Biogenic synthesis of floral-shaped gold nanoparticles using a novel strain, *Talaromyces flavus*. *Annals of Microbiology*, vol. 64, no. 3, pp. 1055-1063. http://dx.doi.org/10.1007/s13213-013-0744-4.

RAJAKUMAR, G. and RAHUMAN, A.A., 2011. Larvicidal activity of synthesized silver nanoparticles using Eclipta prostrata leaf extract against filariasis and malaria vectors. *Acta Tropica*, vol. 118, no. 3, pp. 196-203. http://dx.doi.org/10.1016/j.actatropica.2011.03.003. PMid:21419749.

SAJJADI, A., 2014. The effect of synthetic nanofertilizer on tobacco collar rot. Proceedings of the 21st Iranian Plant Protection Congress, 1-4 September 2014, Urmia, Iran. Tehran, Iran: Agricultural Research,Education and Extension Organization (AREEO), p. 135.

SALUNKHE, R.B., PATIL, S.V., PATIL, C.D. and SALUNKE, B.K., 2011. Larvicidal potential of silver nanoparticles synthesized using fungus *Cochliobolus lunatus* against *Aedes aegypti* (Linnaeus, 1762) and *Anopheles stephensi* Liston (Diptera; Culicidae). *Parasitology Research*, vol. 109, no. 3, pp. 823-831. http://dx.doi.org/10.1007/s00436-011-2328-1. PMid:21451993.

SARGIN, S., GEZGIN, Y., ELTEM, R. and VARDAR, F., 2013. Micropropagule production from *Trichoderma harzianum* EGE-K38 using solid-state fermentation and a comparative study for drying methods. *Turkish Journal of Biology*, vol. 37, no. 1, pp. 1-8.

SCHUSTER, A. and SCHMOLL, M., 2010. Biology and biotechnology of Trichoderma. *Applied Microbiology and Biotechnology*, vol. 87, no. 3, pp. 787-799. http://dx.doi.org/10.1007/s00253-010-2632-1. PMid:20461510.

SEEFELDT, S.S., PETERS, E., ARMSTRONG, M.L. and RAHMAN, A., 2001. Cross-resistance in chlorsulfuron-resistant chickweed (*Stellaria media*). *New Zealand Plant Protection*, vol. 54, no. 1, pp. 157-161. http://dx.doi.org/10.30843/nzpp.2001.54.3714.

SHABGAH, A.G., QASIM, M.T., MOSTAFAVI, S.M., ZEKIY, A.O., EZZATIFAR, F., AHMADI, M., HAFTCHESHMEH, S.M. and NAVASHENAQ, J.G., 2021. CXC chemokine ligand 16: a Swiss army knife chemokine in cancer. *Expert Reviews in Molecular Medicine*, vol. 23, p. e4. http://dx.doi.org/10.1017/erm.2021.7. PMid:33880989.

VASANE, S.R. and KOTHARI, R.M., 2008. An integrated approach to primary and secondary hardening of banana Var. Grand Naine. *Indian Journal of Biotechnology*, vol. 7, no. 2, pp. 240-245.

WAN-JUN, S., WEI-WEI, S., SAI-YAN, G., YI-TONG, L., YONG-SONG, C. and PEI, Z., 2010. Effects of nanopesticide chlorfenapyr on mice. *Toxicological and Environmental Chemistry*, vol. 92, no. 10, pp. 1901-1907. http://dx.doi.org/10.1080/02772248.2010.483758.

WHIPPS, J.M., 2001. Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany*, vol. 52, suppl. 1, pp. 487-511. http://dx.doi.org/10.1093/jxb/52.suppl_1.487. PMid:11326055.

WOKOMA, E.C.W., 2008. Preliminary report on diseases of tomato in Choba, Rivers State.*Journal of Applied Sciences and Environmental Management*, vol. 12, suppl. 3, pp. 117-121.

ZHANG, Q., HUA, G. and ADANG, M.J., 2015. Chitosan/DsiRNA nanoparticle targeting identifies AgCad1 cadherin in *Anopheles gambiae* larvae as an *in vivo* receptor of Cry11Ba toxin of *Bacillus thuringiensis* subsp. *jegathesan*. *Insect Biochemistry and Molecular Biology*, vol. 60, no. 1, pp. 33-38. http://dx.doi.org/10.1016/j.ibmb.2015.03.001. PMid:25758367.