Effectiveness of the chemical stabilizers of *Talaromyces flavus* in biological control of tomato and greenhouse cucumber vascular wilt disease

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Abstract: Fungal antagonist, *Talaromyces flavus*, is one of the most important biological agents of soil-borne fungal diseases including Verticillium and Fusarium wilt. In this study, to increase the effectiveness of *T. flavus* isolates obtained from greenhouse cucumbers and field grown tomatoes five chemical stabilizers were evaluated. Based on the results of previous studies, the most effective substrate for the growth, sporulation and stability of *T. flavus* isolates related to the above-mentioned plants was a mix of rice bran and peat-moss. Different chemical stabilizers were mixed with the above-mentioned substrate containing spore suspensions of various *T. flavus* isolates. For each plant, a completely randomized experiment was conducted under greenhouse conditions with seven treatments and three replications. The results of this study indicated that treatments containing sodium nitrate and D-cycloserine were more effective than those containing other stabilizers. The overall results of this study suggest that the use of some chemical stabilizers may enhance the biocontrol potential of fungal antagonists in controlling different plant diseases including Verticillium and Fusarium wilt.

Key words: bioformulation, *Fusarium oxysporum*, greenhouse cucumber, *Talaromyces flavus*, tomato, *Verticillium dahliae*

Introduction

There have been abundant reports during the last decade about biological fungicides by solid substrates and optimizing production procedures (Pascual *et al*. 1999; Budge and Whipps 2001; Schuster and Schmoll 2010; Damaso *et al*. 2012; Sargin *et al*. 2013). For instance, Pascual *et al*. (1999) showed the solid biological fungicide containing the fungus *Epicoccum nigrum* on wheat. Research on the effect of alcoholic solutions containing glycerol, mannitol and arabitol on sporulation of this fungus found that glycerol caused the most significant increase of sporulation.

Sargin *et al*. (2013) observed that *Trichoderma harzianum* EGE-K38 increased the biological efficiency of biological fungicides when compared with diverse desiccation methods used for this fungicide. The results of other studies have shown that the application of compounds, including minerals such as manganese, iron, zinc and phosphorus in biological fertilizers, led to an increase in their stability (Vasane and Kothari 2008; Lee and Lee 2009). So far, biological preparations such as Ketomium® containing *Chaetomium globosum* and *Chaetomium cupreum*, Promote® containing *T. harzianum* and *T. viride*, Solid Gard® containing *Gliocladium virens*, Trichodex® containing *T. harzianum*, *Pisolithus tinctorius* and *Glomus intraradices*, Trichodermin® containing *T. harzianum*, and Protus WG® containing *Talaromyces flavus* have been commercially registered overseas (Merwel *et al*. 1974; Koch 1999; Kaewchai *et al*. 2009).

With regard to the importance of soil-borne diseases such as Verticillium wilt, Fusarium wilt, Pythium root rot and seedling damping-off in most crops and greenhouse products including tomato and greenhouse cucumber in Iran (Ghaderi 2011; Sharzehi *et al*. 2011), as well as the role played by *T. flavus* fungus as an effective antagonist against soil-borne fungal pathogens including *Fusarium oxysporum*, *Verticillium albo-atrum*, *Verticillium dahliae* and *Rhizoctonia solani* (Madi *et al*. 1992; Madi *et al*. 1997; Duo-Chuan *et al*. 2005; Haggag *et al*. 2006; Ashraf and Khan 2007), serious measures have been undertaken to isolate the diverse, aforesaid antagonist isolates from the main cultivation areas of certain crops (Naraghi *et al*. 2013).

Numerous laboratory and greenhouse studies have determined the antagonist impact of these isolates against the mentioned pathogenic agents. The most effective isolates have been effective in terms of controlling the pathogenic agent of each product. Biological fungicides which are affected by different *T. flavus* isolates in controlling the aforesaid pathogenic agents were prepared for each product. For large-scale application of these fungicides appropriate technical knowledge is necessary for mass

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production. Marketing and commercialization of these fungicides are therefore some of the most important issues for manufacturers (Alimi *et al*. 2006; Husen *et al*. 2007; Kaewchai *et al*. 2009; Pereira *et al*. 2009). According to recent research increased efficiency and stability of these types of fungicides are the most crucial factors in marketing and commercialization (Kaewchai *et al*. 2009; Mukhopadhyay and Maiti 2009; Ghaderi-Daneshmand *et al*. 2012).

Organic and inorganic stabilizing compounds for different *T. flavus* metabolites have been determined (Yu and Chang 1987; Cimarelli *et al*. 2001; Matos *et al*. 2012). So the present study aims to optimize the *T. flavus* bioformulation through the utilization of these stabilizers and select the most effective one in terms of its biological control capability of bioformulation for seedling damping-off disease in tomato and greenhouse cucumber plants.

Materials and Methods

Development of *Talaromyces flavus* bioformulations

According to Naraghi *et al*. (2013), the most effective *T. flavus* isolates include, respectively TF-To-V-24 (isolate No. 24 obtained from the soil of Varamin tomato fields) and TF-Cu-V-60 (isolate No. 60 obtained from the soil of Varamin cucumber greenhouses). These were utilized to control Fusarium wilt and Verticillium wilt of tomato and greenhouse cucumber plants.

The following modified method of Naraghi *et al*. (2010) was applied to prepare the required bioformulations from related isolates (TF-To-V-24 and TF-Cu-V-60) was used.

A specified amount of rice bran was steeped in hot water (30–35°C) for 24 h, after which it was spread and dried on large filter paper. In the next step, 200 g of rice bran and 50 g of cleaned peat soil was sterilized in cellophane bags in an autoclave (1 atm, 120°C for 15 min). Afterwards, a suspension containing 20 ml of sterilized distilled water and four pieces of 1 cm culture medium, 10 days old, and belonging to the related isolate, was poured in to cellophane bags to prepare the inoculum of each isolate. The stabilizer compounds containing aminophenol, D-cycloserine, magnesium sulfate, carboxymethyl cellulose and sodium nitrate in proportion to treatment were added to the growth medium on the basis of supplements (10 ml of supplement solution with 20 g · l^{-1} for 250 g of every medium). For isolate growth, the cellophane bags were incubated at 30°C for almost a month and a half to two months during which 20 ml of distilled water was added for better moisture in case the contents were dried. After this period, the content of each cellophane bag was spread on filter paper to be desiccated, added to soil and utilized as bioformulation in greenhouses. In order to add the antagonist bioformulation to soil, on the basis of 2 × 10^7 cfu · g^{-1} of soil, the amount of each bioformulation to be added to the soil for each treatment was determined through the calculation of the number of spores per gram of bioformulation using hemocytometer lam (Aziz *et al*. 1997).

Preparation of pathogenic agents

The *V. dahliae* and *F. oxysporum* isolates were applied. The pathogenicity of these isolates on tomato and greenhouse cucumber has been shown (Naraghi *et al*. 2012). The isolates utilized for tomato included: VD-Co-P-G-22 (*V. dahliae* isolate obtained from the cotton stem in the Gorgan field with an infection index of 48%), and FO-To-S-V-1 (*F. oxysporum* isolate obtained from the soil of the Varamin tomato field with 30% disease severity). The isolates utilized for greenhouse cucumber included: VD-Co-P--G-22 (*V. dahliae* obtained from the cotton stem in the Gorgan field) and FO-Cu-S-V-1 (*F. oxysporum* isolate obtained from the soil of the cucumber greenhouse in Varamin).

Preparation and inoculation of *Verticillium dahliae* inoculum

The *V. dahliae* inoculum was prepared according to the aforesaid method of Sprink and Rowe (1989). For this purpose, some subcultures of *V. dahliae* were prepared. After 14 to 17 days, the microsclerotia of *V. dahliae* were germinated on culture medium [Czapek Solution Agar or Potato Dextrose Agar (PDA)]. Then the surfaces of the culture media were washed three times to remove the mycelium and conidia parts and the culture media containing microsclerotia was combined with a specified volume of dry sterile soil. For inoculation of the obtained inoculum, serial dilution was made from the mentioned inoculum. One milliliter of each dilution was cultured on Petri dishes containing the culture medium of streptomycin and agar sulfate-alcohol. The related Petri dishes were kept for 7 to 10 days at approximately 22°C. By observation of *V. dahliae* colonies on the medium surface and through estimation of the numbers of countable colonies in the Petri dish, the number of microsclerotia per gram of inoculum was obtained. In the following stage, based on the 200 number of microsclerotia per gram of soil, the required amount of inoculum for the affected treatments was specified (Naraghi *et al*. 2003).

Preparation and inoculation of *Fusarium oxysporum* inoculum

The preparation of inoculum and inoculation of *F. oxysporum* pathogenic agent was conducted simultaneously with planting based on a modified method of Khalil *et al*. (2003). For this purpose, a 500 ml flask containing 100 g of corn seed and 80 ml of town water were placed in an autoclave for 30 min. After transferring two to three 5 ml cultured parts for one week from fungus to the flask and complete mixing of its content, the flask was kept in an incubator at 30°C for three weeks. When the corn seed surfaces were completely covered with mycelium fungus, the flask content was spread out at laboratory temperature and utilized as apathogenic agentinoculum. With the calculation of microconidia numbers per gram of inoculant by hemocytometer lam, a certain amount was added to pot soil which was defined as a 10^7 cfu · g^{-1} of soil (Anitha and Rabeeth 2010).

Evaluation of the efficacy of bioformulations in controlling Verticillium and Fusarium wilt in tomatoes and greenhouse cucumbers

Four experiments were carried out separately for tomato and greenhouse cucumber plants. Two pathogenic agents, *V. dahliae* and *F. oxysporum*, were also studied. Each experiment was conducted with a completely randomized design in seven treatments and three replications. The treatments of each experiment included the *T. flavus* inoculants affected by five different stabilizers (aminophenol, D-cycloserine, magnesium sulfate, carboxymethyl cellulose and sodium nitrate) as well as the healthy and infected control experiment. The final assessment of the treatment related to each experiment was based on disease index. Data were analyzed by ANOVA (Analysis of Variance) using the MSTAT-C statistical software, while Duncan separated means as multiple range tests.

Evaluation method of Verticillium wilt

The evaluation of disease was performed 45 days after sowing by determining the infection index of Verticillium wilt according to the following formula (Yakutin 1972):

$$\text{I.I.} = \frac{1n + 2n + 3n + 4n}{4N} \times 100,$$

where:
I.I. – infection index
1 – first grade leaf = symptoms of chlorosis and necrosis for a quarter of leaf
2 – second grade leaf = symptoms of chlorosis and necrosis for two quarters of leaf
3 – third grade leaf = symptoms of chlorosis and necrosis for three quarters of leaf
4 – fourth grade leaf = symptoms of chlorosis and necrosis for three quarters or the entire leaf
n – number of leaves related to each grade
N – total number of leaves on a bush

Evaluation method of Fusarium wilt

The evaluation of Fusarium wilt disease was conducted three weeks after inoculation by determining the percentage of disease severity according to Hao *et al*. (2005). The following 0 to 5 scales was used:
0 – without symptoms
1 – leaf chlorosisand plant wilt less than 25%
2 – leaf chlorosisand plant wilt from 25 to 50%
3 – leaf chlorosisand plant wilt from 51 to 75%
4 – leaf chlorosisand plant wilt from 76 to 100%
5 – dead or fully destroyed plant

$$\text{Disease severity percent} = \frac{\sum(n_i \times v_i)}{N \times V} \times 100,$$

where: n_i = the number of the samples with the same olisease scale; v_i = the olisease scale for every sample; N = total samples in each replications; V = maximum olisease scale.

Results

Calculated amount of *Talaromyces flavus* bioformulation for pot soil

The required amount of *T. flavus* antagonist isolate (TF-To-V-24 for tomato and TF-Cu-V-60 isolate for greenhouse cucumber) in each pot containing 3 kg soil, based on 2×10^7 cfu $\cdot$ g^{-1} of soil (mentioned in the Materials and Methods section) and calculating 6×10^9 cfu $\cdot$ g^{-1} of bioformulation prepared from *T. flavus* isolates (TF-To-V-24 and TF-Cu-V-60) was determined. In this regard, the amount of 10 g of TF-To-V-24 or TF-Cu-V-60 inoculum isolate was defined for each pot related to tomato and greenhouse cucumber plants.

Calculated amount of *Verticillium dahliae* pathogenic inoculum for pot soil

The required amount of *V. dahliae* pathogenic inoculum for each pot containing 3 kg soil, based on 200 numbers of microsclerotia per gram of soil (mentioned in the Materials and Methods section) and calculating 3×10^4 per gram of inoculant prepared from *V. dahliae* isolate (VD-Co-P-22) was prepared. In this regard, the amount of 20 g of VD-Co-P-G-22 inoculum isolate was defined for each pot related to tomato and greenhouse cucumber plants.

Calculated amount of *Fusarium oxysporum* pathogenic inoculum for pot soil

The required amount of *F. oxysporum* (FO-To-S-V-1 for tomato and FO-Cu-S-V-1 isolate for greenhouse cucumber) inoculum in each pot containing 3 kg soil, based on 2×10^7 cfu $\cdot$ g^{-1} of soil and calculating 2×10^9 cfu $\cdot$ g^{-1} of inoculum was determined. In this regard, the amount of 15 g of FO-To-S-V-1 or FO-Cu-S-V-1 inoculum was defined for each pot related to tomato and greenhouse cucumber plants.

Evaluation of the efficacy of bioformulations in controlling Verticillium and Fusarium wilt in tomato and greenhouse cucumber

The results of this section for each of the pathogenic agents of tomato and greenhouse cucumber plants are presented below.

Tomato Verticillium wilt

The experiment on the impact of *T. flavus* inoculum which included the different stabilizers on tomato Verticillium wilt was significant at 1% probability level. A comparison of mean disease index levels with different treatments showed that all indices were categorized into four statistical groups. All the treatments showed significant decreases in disease index when compared with the infected control group. Among these treatments, the lowest disease index belonged to the treatment containing the sodium nitrate stabilizer. There was no significant statistical difference at 1% probability level among other treatments (Table 1).

Table 1. A comparison of tomato Verticillium wilt indices in bioformulation treatments containing different stabilizers in the greenhouse experiment

Treatment	Disease index
Talaromyces flavus bioformulation with aminophenol stabilizer	6.17 ab
T. flavus bioformulation with D-cycloserine stabilizer	4.21 ab
T. flavus bioformulation with magnesium sulfate stabilizer	5.04 ab
T. flavus bioformulation with carboxymethyl cellulose stabilizer	4.13 ab
T. flavus bioformulation with sodium nitrate stabilizer	3.99 b
Infected control	11.32 a
Healthy control	0.00 c

Treatments marked by the same letter(s) are not significantly different ($p > 0.01$)

Tomato Fusarium wilt

The experiment on the effect of *T. flavus* bioformulations which included the different stabilizers on tomato Fusarium wilt was significant at 1% probability level. A comparison of mean disease severity percent a with different treatments showed that all indices were categorized into six statistical groups. All the treatments, except the one including aminophenol, showed significant decreases in disease severity when compared with the infected control group. Among these treatments, the least disease severity mean, respectively, belonged to the treatments containing the sodium nitrate stabilizer and carboxymethyl cellulose. There was no significant statistical difference at 1% probability level between D-cycloserine and magnesium sulfate (Table 2).

Greenhouse cucumber Verticillium wilt

The experiment on the impact of *T. flavus* bioformulations which included the different stabilizers on tomato Verticillium wilt was significant at 1% probability level. A comparison of mean disease index levels with different treatments showed that all indices were categorized into seven statistical groups. All treatments showed significant decreases in disease index when compared with the infected control group. Among these treatments, the lowest and greatest disease index averages, respectively, belonged to the treatments containing the sodium nitrate stabilizer and the aminophenol stabilizer. Other treatments containing stabilizer, ordered from most to least efficient in controlling the Verticillium disease were as follows: D-cycloserine, carboxymethyl cellulose and magnesium sulfate (Table 3).

Table 2. A comparison of tomato Fusarium wilt severity in bioformulation treatments containing different stabilizers in the greenhouse experiment

Treatment	Disease severity [%]
Talaromyces flavus bioformulation with aminophenol stabilizer	23.33 a
T. flavus bioformulation with D-cycloserine stabilizer	15.45 abc
T. flavus bioformulation with magnesium sulfate stabilizer	19.58 abc
T. flavus bioformulation with carboxymethyl cellulose stabilizer	12.49 bc
T. flavus bioformulation with sodium nitrate stabilizer	9.99 c
Infected control	18.74 a
Healthy control	0.00 d

Treatments marked by the same letter(s) are not significantly different ($p > 0.01$)

Table 3. A comparison of cucumber Verticillium wilt indices in bioformulation treatments containing different stabilizers in the greenhouse experiment

Treatment	Disease index
Talaromyces flavus bioformulation with aminophenol stabilizer	54.03 ab
T. flavus bioformulation with D-cycloserine stabilizer	31.31 cd
T. flavus bioformulation with magnesium sulfate stabilizer	46.48 abc
T. flavus bioformulation with carboxymethyl cellulose stabilizer	39.70 bc
T. flavus bioformulation with sodium nitrate stabilizer	18.27 d
Infected control	69.23 a
Healthy control	0.00 e

Treatments marked by the same letter(s) are not significantly different ($p > 0.01$)

Table 4. A comparison of cucumber Fusarium wilt severity in bioformulation treatments containing different stabilizers in the greenhouse experiment

Treatment	Disease severity [%]
Talaromyces flavus bioformulation with aminophenol stabilizer	71.66 a
T. flavus bioformulation with D-cycloserine stabilizer	40.83 b
T. flavus bioformulation with magnesium sulfate stabilizer	48.33 b
T. flavus bioformulation with carboxymethyl cellulose stabilizer	48.32 b
T. flavus bioformulation with sodium nitrate stabilizer	37.50 b
Infected control	79.99 a
Healthy control	0.00 c

Treatments marked by the same letter(s) are not significantly different ($p > 0.01$)

Greenhouse cucumber Fusarium wilt

The experiment on the effect of *T. flavus* bioformulation with different stabilizers on greenhouse cucumber Fusarium wilt was significant at 1% probability level. A comparison of mean disease severity percent with different treatments showed that all the averages were categorized into three statistical groups. All the treatments, except the one containing aminophenol, showed significant decreases in disease severity when compared with the infected control group. Among other treatments containing stabilizers, there was no significant statistical difference at 1% probability level in terms of disease severity percent (Table 4).

Discussion

The overall results of this study suggest that the use of some chemical stabilizers may enhance the biocontrol potential of fungal antagonists in controlling different plant diseases including Verticillium and Fusarium wilt.

In our research the application of *T. flavus* antagonist fungus inoculum including several chemical stabilizers such as sodium nitrate, carboxymethyl cellulose, D-cycloserine and magnesium sulfate led to significant decreases of certain important soil-borne fungus diseases such as seedling damping-off in tomato and greenhouse cucumber. No precise information is available on the application of additive substances to biological compounds in order to increase their stability. But studies conducted to date indicate that increased efficiency and stability of biological compounds are some of the most important factors in marketing and commercialization of these products (Mukhopadhyay and Maiti 2009; Kaewchai *et al*. 2009; Ghaderi-Daneshmand *et al*. 2012).

The results of the previous research showed that some chemical compounds including sodium nitrate, potassium phosphate, magnesium sulfate, L-asparagine, L-sorbose caused the growth inhibition of the several soil-borne fungal pathogenic agents such as *Verticillium* and *Fusarium* (Ausher *et al*. 1975; Christen 1982; Hadar and Katan 1989; Veverka *et al*. 2007). The present study showed that *T. flavus* bioformulation containing sodium nitrate used to control the soil-borne fungus diseases being researched (*Rhizoctonia* seedling damping-off) was effective in tomato and greenhouse cucumber. Thus, according to the description above, such a result was not unexpected.

On the other hand, an osmotic stabilizer such as sodium nitrate was reported as the stabilizing compound for the chitinase enzyme (Gavanji *et al*. 2013; Patil and Jadhav 2015). Such a compound could therefore play an important role in the maintenance of the metabolite related to the mycoparasitism mechanism of *T. flavus* which is a chitinase enzyme (Inbar and Chet 1995). The *T. flavus* present in inoculum has shown desirable efficiency in disease control due to the above-mentioned metabolite.

In the present research, no differences were observed in tomatoes and greenhouse cucumbers in terms of the impact of two diverse *T. flavus* inoculants with carboxymethyl cellulose and D-cycloserine stabilizers to control the diseases under study. The results showed that *T. flavus* bioformulations containing carboxymethyl cellulose were more successful than *T. flavus* bioformulation containing D-cycloserine stabilizer to control the aforesaid disease in tomato. But the reverse condition occurred for greenhouse cucumber. To understand this, it is necessary to mention the relation between effective metabolites of different *T. flavus* isolates to control the pathogenic agents with host plants.

Among the non-volatile *T. flavus* metabolites, including glucose oxidase, xilodase and betagalactosidase, the glucose oxidase enzyme played a vital role in controlling major herbal pathogenic agents (Jat and Agalave 2013). The activity of this enzyme occurred in the presence of glucose existing in host plant root exudates, thereby leading to the destruction of pathogenic fungal and bacterial populations by producing toxic compounds with hydrogen peroxide (Kim *et al*. 1989). We expect that the activity of the related enzymes are increased for *T. flavus* isolates which are found around the roots with root secretions rich in sugar compounds. On the contrary, the activity level of glucose oxidase obtained from the rhizospheres of such plants is significantly higher than the *T. flavus* isolates related to plants with root secretions poor in glucose compounds.

The study has been applied to related rhizospheres for both tomato and greenhouse cucumber plants. The data show that, due to the existence of more glucose compounds in root secretions of tomato compared to green-

house enzymes, the activity of glucose oxidase enzyme in isolates related to tomato is increased and makes use of its accessible glucose compounds such as carboxymethyl cellulose. On the other hand, D-cycloserine and carboxymethyl cellulose are considered to be two non-volatile metabolite stabilizers (Matos *et al*. 2012). For *T. flavus* isolates related to greenhouse cucumber (a plant with root secretions poor in glucose compounds), D-cycloserine increased the durability of other non-volatile metabolites such as xilodase and betagalactosidase to control the pathogenic agents.

Moreover, the findings showed that *T. flavus* bioformulation containing aminophenol and magnesium sulfate, compared to inoculums with other stabilizers in terms of efficiency to control the diseases under study, had poor performance. Based on the reports about the introduction of aminophenol as a volatile metabolite stabilizer of *T. flavus* containing ethylene, hydrogen, cyanide, alcohol and aldehyde compounds (Cimarelli *et al*. 2001) and recognition of magnesium sulfate as a chitinase enzyme (a major metabolite of mycoparasitism) (Yu and Chang 1987), the results of the present paper could be discussed as follows. Previous studies have shown that alcoholic compounds, such as ethanol, improved the mycelium growth in several soil-borne fungi such as *Fusarium*, *Pythium* and *Rhizoctonia* (Vincelli and Beaupre 1989; Menz *et al*. 2011). With regard to the placement of aminophenol compounds in alcoholic groups, the lack of efficiency of *T. flavus* inoculum containing aminophenol stabilizer would be justified. On the other hand, the low efficiency of *T. flavus* bioformulation containing magnesium sulfate in certain pathogenic agents could be due to the incompatibility of *T. flavus* antagonist with the mentioned compounds. The studies of Hiscox *et al*. (2015) and Shanmugam *et al*. (2010) in this regard showed that it is considered as stress for fungal growth under certain chemical conditions. It is supposed that it happens before *T. flavus* bioformulation enters the soil, though magnesium compounds in certain cases caused the weak growth of *T. flavus*.

We conclude that inoculums containing *T. flavus* isolates related to tomato and greenhouse cucumber simultaneously with sodium nitrate stabilizers and D-cycloserine exhibited desirable efficiency in controlling the Verticillium and Fusarium wilts in the aforesaid products.

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