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Biological control of plant fungal diseases using volatile substances of *Streptomyces griseus*

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ABSTRACT

Streptomyces griseus is a useful bacteria that produces many secondary metabolites and volatile compounds. In this project, antifungal activity of volatile substances derived from Streptomyces griseus against Penicillium chrysogenum and Botrytis cinerea was studied in vitro. The compositions of volatiles were also determined by gas chromatography comb with mass spectrometry analysis. Fungal spore germination and mycelium growth of both P.chrysogenum and B.cinerea cultures were significantly suppressed in the presence of the volatiles. Gas chromatography comb with mass spectrometry analysis results showed that twenty volatile compounds were identified in one week old diphasic cultures (Tryptic Soy Agar and Tryptic Soy Broth) of S.griseus. The volatile compounds were chemically grouped into organic acids, alcohol, alkanes, alkenes, alkens and ketones. The most abundant compounds in volatile of S.griseus were Phenol,2-methyl-5-1methylethyl(Carvacrol). Chemicals of less abundant were Isocyclocitral, Benzene,1,2-dimethoxy -4-1-methylethenyl. The antifungal activity of S.griseus cultures can be attributed to Carvacrol, Dimethyl sulfoxide (DMSO), Cyclohexanol, Naphthalene. The volatile substances of S.griseus have a potential for using as a biofumigant to control plant fungal diseases.

Key words: *Streptomyces griseus, Botrytis cinerea, Penicillium chrysogenum*, Volatile substances, antifungal.

INTRODUCTION

Streptomyces are gram-positive, aerobic and branching bacteria which have been widely examined by researchers across the globe. They are able to produce useful secondary metabolites including different antibiotics[15].Bacterial volatile substances are among the secondary metabolites which have been recently investigated[12-18].Volatile substances produced by bacteria are substances with molecular weight of <300 Da, low polarity, and high vapor pressure [27]. Bacterial volatile substances have been successfully recognized by gas chromatography comb with mass spectrometry (GC-MS). For example, more than 120 various substances have been recognized in actinomycetes including Alkanes, Alkenes, Alkens, Alcohols, Ketones, Aldehydes, Acids and Esters[30]. Volatile substances derived from *Streptomyces sp*. and other species of actinomycetes prevent mycelium growth and inhibit spore germination of different fungi [19,4]. Cyclohexanol, decanol, 2-ethyl-1-hexanol, nonanol, benzothiazole and

dimethyl trisulfide are important compounds that inhibit spore germination and mycelium growth of *Sclerotinia sclerotiorum* [12]. Anitha and Rabeeth showed chitinase and lytic enzymes of *S.griseus* can control some fungal plant diseases like *Fusarium oxysporum*, *Alternaria alternata*, *Rhizoctonia solani* and *F.*solani[4, 5].Volatile substances of *Streptomyces griseus* reduces spore germination of *Gleosporium aridum* which subsequently lead to faster formation of sclerotinia of *R.solani* and *S.cepivorum*. Volatile substances of *Streptomyces platensis* also reduced the growth of *R.solani*, *S.sclerotiorum* and *B.cinerea* and reduced the disease level of leaf blight/seedling blight in rice, leaf blight in oilseed rape, and fruit rot in strawberry [23, 31]. In another research, effects of volatile substances of *S.globisporus* were examined on spore germinating and mycelium growth *Penicillium italicum* and infected fruits. Among 41 volatile substances of this bacterium, Dimethyl disulfide and Dimethyl trisulfide have high inhibiting effects against fungus [21]. Volatile substances of various species of *Streptomyces*, have high potential in biological control. In this project, the effects of *S.griseus* volatile substances were examined on cilium growth and spore germination of *B.cinerea* and *P.chrysogenum*, and finally volatile substances of *S.griseus* were collected and analyzed.

MATERIALS AND METHODS

Microorganisms and cultures media

S.griseus was prepared in Persian Type Culture Collection No. PTCC 1455. Lyophilized ampoules were inoculated under sterile conditions on Yeast extract-Malt extract (YMA) media and were incubated for five days at 30°C. For short storage, *S.griseus* was inoculated in the test tube slants of YMA and stored at 4°C. *P.chrysogenum (*PTCC5035) was also prepared in Persian Type Culture Collection and *B.cinerea* was prepared from Academic Scientific Research of Kerman and was inoculated on Malt extract Agar (MEA) media. Fungi were incubated at 27 $^{\circ}$C for seven days. Stock cultures of each isolated species were maintained on PDA at 4°C.

Preparation of spore suspension

To prepare spore suspension, 20 ml of 5% (v/v) of Tween 80 was added to MEA culture media. *B.cinerea* and *P.chrysogenum* were inoculated for five days at 30°C in slant glass tubes, subsequently, they were shaken gently. Suspension was gently passed through three layer cheese clothes and was diluted using sterile water. Dilution of 10^5 spore/ml was selected as suitable dilution, using hemocytometer.

Inhibiting effect of volatile substances of *S.griseus* on mycelium growth

To evaluate the effect of volatile substances of *S.griseus* on mycelium growth suppression of *B.cinerea* and *P.chrysogenum* one technique were used. In this method, *S.griseus* was superficially cultured on TSA media in 9 cm plates by swap, then a circle with the diameter of 3cm was removed from the TSA culture media and a circle with the diameter of 2cm from MEA media was placed in the previous ring. The middle of MEA media was inoculated with 2 mm plug mycelium of *B.cinerea* and *P.chrysogenum*. Plates were closed immediately with Parafilm. All samples were inoculated for seven days at 30°C. For each treatment, there were four replicates, and the experiments were repeated twice.

Inhibiting effect of volatile substances of S.griseus on spore germination

To examine the effect of volatile substances of *S.griseus* on spore germination of *B.cinerea* and *P.chrysogenum*, the inverse double technique was used. In this method, 200 µl of spore suspension (10^5 spore/ml) of *P.chrysogenum* and *B.cinerea* were inoculated on MEA petri plates. A petri dish was superposed on another, so the bottom of a plate became the top lid of another containing *S.griseus* culture on TSA. The two dishes were sealed together by Parafilm. Spore germination were observed under a microscope after 24, 48, and 72 h. The inhibition percentage of spore germination was calculated as compared to the control. There were four replicates for each treatment and the experiments were repeated twice.

Collection and GC-MS analysis

To evaluate volatile substances of *S.griseus* 20 ml TSA culture media was added to solid and liquid phases of the biphasic blood culture bottle. After the bottles and culture media were sterilized, bottles were placed horizontally in order to the solid phase of culture media forms on the bottle wall. After that, 20 ml of Tryptic Soy Broth (TSB) culture media was added to the glass in sterile condition so that the media had both solid and liquid phases. Diphasic media was inoculated by *S.griseus*. Samples were incubated at 30 $^{\circ}$C for ten days (Fig. 2). Gas chromatography was performed using a 5975C series GC with a flame ionization detector (7890A) (Agilent Technology, USA), in the Department of Chemistry, Islamic Azad University of Kerman. Then, the syringe was pulled out from the diphasic

media bottles and inserted into a gas chromatograph. A 30m×250µm×0.25µm column was used for separation of the volatiles. The working temperature for the volatile separation column was programmed as: 40 °C for 2 min, 150°C for 2 min and 280 °C for 10 min. The carrier gas was Helium with a flow rate of 1ml/min. Standard wiley and National Institute of Standards and Technology (NIST) Mass Spectral (version 2.0) were used to identify the volatiles. Analysis of separation was replicated twice.

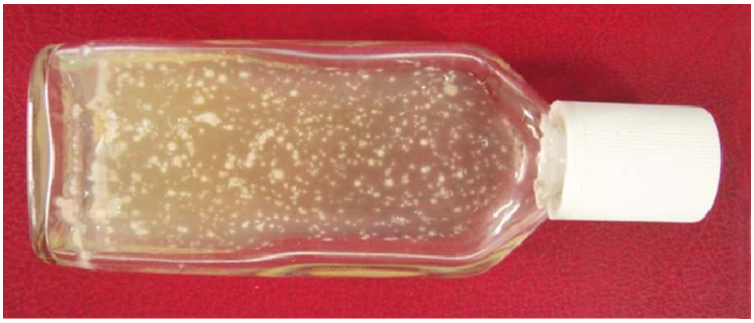

Fig1.To evaluate volatile substances of *S.griseus*, solid and liquid phases of the biphasic blood culture bottle were used

Data analysis

The data were subjected to analyses of variance (ANOVA) using SPSS 13.0 software for windows (SPSS Inc., Chicago, USA). Mean comparisons were performed by Fisher's Protected Least Significant Difference (LSD) test ($P< 0.05$).

RESULTS

Inhibitory effect of volatile substances of S.griseus on mycelia growth of B.cinerea and P.chrysogenum

In the new technique, the average colony diameter was 9 mm for *B.cinerea* and 5.5 mm for *P.chrysogenum*, following 24 h incubation in the presence of *S.griseus* at 30°C (Fig 2). However, in the control experiment (without *S.griseus* cultures), the average colony diameter was 20.75 mm for *B.cinerea* and 8.1 mm for *P.chrysogenum* (Fig 2). The average of the colony diameter *of B.cinerea* and *P.chrysogenum* was14.17 and 9.6 mm for *B.cinerea* and *P.chrysogenum* following 7 day incubation with *S.griseus* cultures, respectively. These amounts were measured 40.75 mm and 16.28 mm for *B.cinerea* and *P.chrysogenum* respectively (without *S.griseus* cultures). So volatile substances of *S.griseus* significantly inhibit the mycelium growth of the studied fungi ($P< 0.001$ for *B.cinerea* and $P< 0.01$ for *P.chrysogenum*).

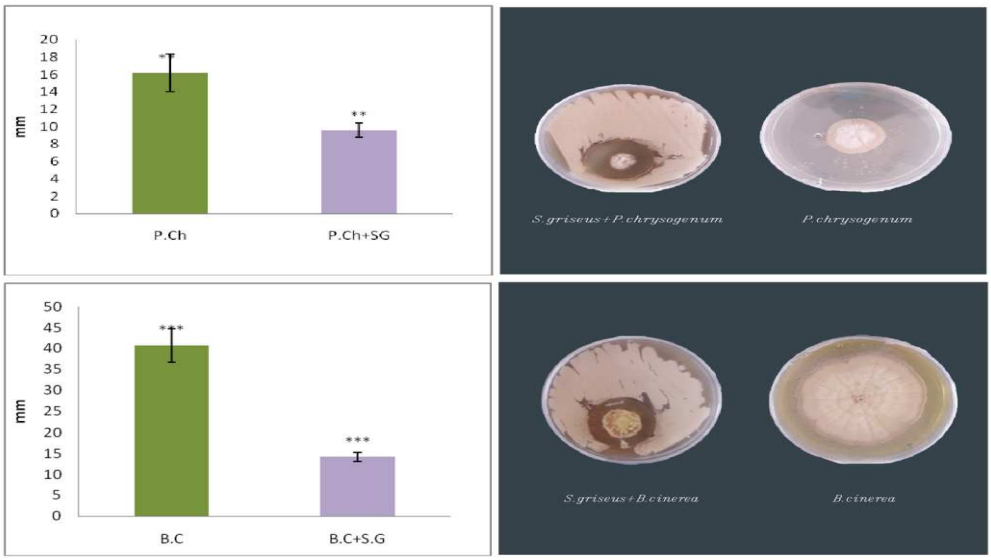

Fig 2. Antifungal activity of *S.griseus* volatiles. Mycelial growth of *B.cinerea* and *P.chrysogenum* were significantly inhibited by volatiles of *S.griseus* as compared to the control (without bacteria)

Inhibitory effect of volatile substances of S.griseus on spore germination of B.cinerea and P.chrysogenum

Spore germination of both fungi were evaluated after 24 h incubation at 30 °C. No spore was germinated in treated samples, while in the control samples germination was started (Fig 3). Means of inhibition of spore germination by the volatiles of *S.griseus* for *B.cinerea* was calculated %92. In the controls plates, all the spores were germinated and colony formation was observed after 48h. Effect of these volatiles on suppression of spore germination of *P.chrysogenum* were stronger than that of *B.cinerea*. No *P.chrysogenum* spore was germinated following 72 h incubation, so the inhibitory effect of volatiles were determined %100 for *P.chrysogenum.*

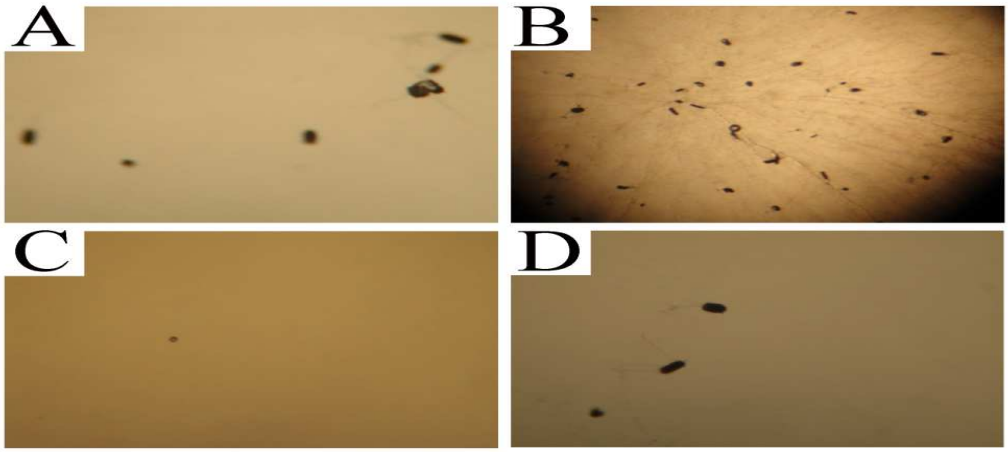

Fig. 3: A: *Botrytis cinerea* spores exposed to the volatiles of *S.griseus* for 72 hr. does not grow to ant mycelium B: Germination of the same spores (*Botrytis cinerea*) not exposed to the volatiles of *S.griseus*. C: *Penicillium chrysogenum* spores exposed to the volatiles of *S.griseus* for 72 hr. D: Germination of the *Penicillium chrysogenum* spores not exposed to the volatiles of *S.griseus*

GC-MS analysis of the volatiles of S.griseus

Analysis of GC-MS identified twenty volatile compounds released from diphasic culture of *S.griseus* (Fig. 4) that were grouped into alcohol, ketones, acids, alkanes, alkens, and alkenes. Among these volatiles, the most abundant was Phenol, 2methyl-5-1-methylethyl(Carvacrol), followed by Dimethyl sulfoxide (DMSO) and 2,6-dihydroxyacetophenone (Table 1.0). Chemicals of less abundance were Isocyclocitral, Benzene, 1,2-dimethoxy -4-1-methylethenyl. Compounds with antifungal activity of *S.griseus* cultures were Carvacrol, DMSO, Cyclohexanol and Naphthalene.

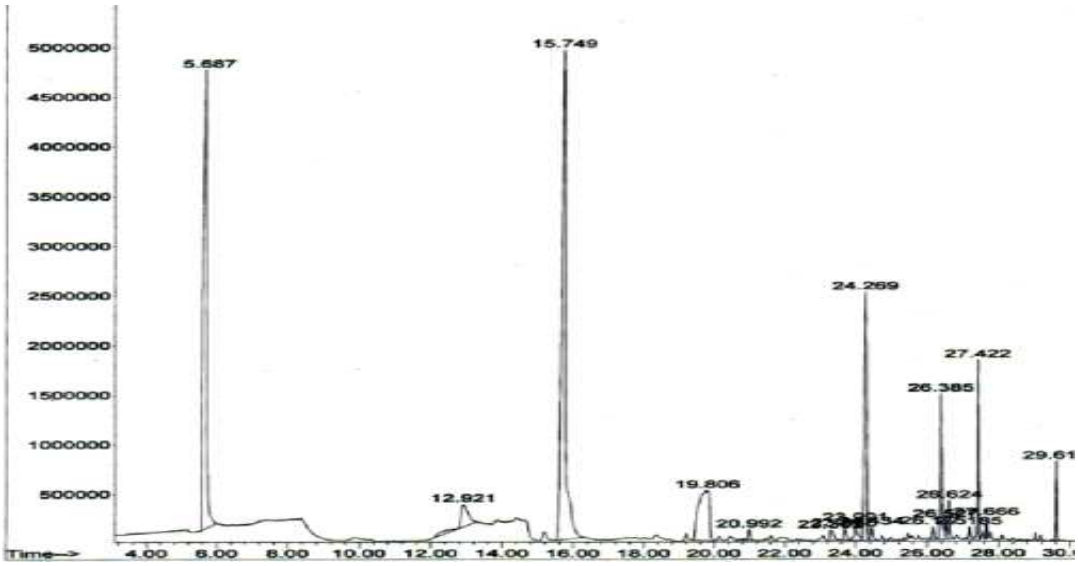

Fig4. A chromatogram of GC-MS analysis of *Streptomyces griseus*, showing twenty volatile organic compounds on diphasic media

Table1.GC-MS analysis of volatile compounds produced by *S.griseus*

RTa(min)	TA(%)	m/z	Possible compound	MW(Da)
5.68	26.06	63	Dimethyl sulfoxide	63
12.92	3.35	73	1-methoxy-4-(1-E-propeny1)benzene	73
15.74	35.24	135	Phenol, 2-methyl-5-(1-methylethyl)	135
19.8	9.37	73	3-Oxa-6-thia-2,7-dosolapctame, 2-2, 7-7-tetramethyl	73
20.99	0.50	161	1H-Cyclopropal [a] naphthalene	161
23.30	0.62	189	Naphtalene	189
23.68	0.45	178	Benzene, 1,2-dimethoxy-4-(1-propenyl)	178
23.99	0.9	150	Cyclohexanol	150
24.269	10.17	73.10	2,6-Dihydroxyacetophenone	73.10
24.43	0.52	207	2,5-di-tert-Butyl-1,4-benzoquinone	207
26.17	0.43	97.10	Thiophen-2-methylamine, N-(2fluorophenyl)	97.10
26.38	3.75	192.10	TRANS-TSOMYRISITICIN	192.10
26.52	0.56	192.10	TRANS-TSOMYRISITICIN	192.10
26.62	0.96	192	Dehydroxy-isocalamendiol	192
27.18	0.32	147.10	Isocyclocitral	147.10
27.42	3.44	73	Ethanedioic acid , bis(trimethylsilyl)	73
27.66	0.44	109	1,4-CIS-1,7-CIS-ACORENONE	109

RT: retention time. TA: total area. MW: molecular weight

DISCUSSION

Streptomyces spp. are famous for producing strong odors. *S.griseus* the accidental discovery of a contaminant isolate that was inhibitory to various phytopathogenic fungi in culture plates led us to investigate whether this isolate could be used in the control *P.chrysogenum* and *B.cinerea*. In this study, 20 volatile compounds from *S.griseus* were identified by GC-MS analysis. Phenol,2-methyl-5-(1-methylethyl)(Carvacrol) was the major component. This volatile compound is also an essential oil of *Origanum vulgar* [13]. The cytotoxic ability of Carvacrol on peroxidant activity can make it an effective antiseptic and antimicrobial agent [5]. Previous investigations showed that Carvacrol has antifungal activities against *Penicillium glabrum*, *P.capisci*, *R.solani*, *F.moniiliforme*, *S.sclerotiorum*, and *Cladosporium herbarum*[2]. Another volatile substance detected in this study was Dimethyl sulfoxide (DMSO) which is used as a routine solvent for antifungal drugs [29].Antifungal activity of DMSO demonstrated by different studies[17]. Elad showed DMSO reduces linear growth of *Botrytis cinerea* isolates, which cause gray mold in potato[11]. In this study, three other volatile substances (Cyclohexanol, Benzaldehyde and Naphthalene) were isolated from *S.griseus*. According to the previous publications, all of these compounds have antimicrobial effects: Cyclohexanol [12], Benzaldehyde and Naphthalene [32]. Significant differences between the volatiles compounds of *S.griseus* and some other Actinomycetes [8-31] were also found. Thus, different species may release similar and dissimilar types of volatile compounds, with various effects[21]. Different bacterial and fungal diseases which may occur at harvest, storage and transport [10] are important causes of postharvest infection in crops. Application of fungicides are routine for postharvest treatment of fruits and vegetables, but their toxicological effects has a risk for human and environmental health[1].At the same time, biological control of postharvest diseases is an interesting object for researchers. Cultures of volatile compounds producing microorganisms can be used in fruit containers [24] and in greenhouses [20]. The Effect of volatile substances of Bacillus pumillus[12],B.subtilis[16] and S. platensis F1 [31] showed that these compounds inhibited mycelium growth and spore germination of *B.cinerea*. The demonstrated effects of volatile substances of *S.griseus*, (92% inhibition of spore germination and mycelium growth) have a potential for the effective control of *B.cinerea*. *P.chrysogenum* was the other fungal studied, causing plant diseases like leaf spot. Freire et al. showed that volatile substances of *B.subtilis* reduced mycelium growth and spore germination of *Penicillium crustomosum* and *P.italicum*[16]. Moore-Landecker and Stotzky showed that microbial volatile could inhibit mycelium growth, spore formation, alternation of colony and morphology of *P.viridicatum*[25]. This activity against spores of *P.italicum* is similar to the fungicidal activity of some plant volatile compounds against *P.expansum* [26].The results of the present study indicated that volatile substances produced by *S. griseus* have a significant effect on the mycelium growth and spore germination of *B.cinerea* and *P.chrysogenum in vitro*. In our study, microscopy picture showed that spores exposed to the volatiles did not germinate after 24 h, even after washing (Fig. 3). In conclusion, the present study showed that the volatiles from *S.griseus* and their components affect *B.cinerea* and *P.chrysogenum* in vitro and could potentially be an effective alternative for the control of postharvest diseases by fumigant action. Further experiments are required to examine the effects of individual compounds and mixtures on plant diseases. Results of this study can be applied for prevention of pathogenicity of *B.cinerea* and *P.chrysogenum* in agricultural activities.

CONCLUSION

Effect of bacterial volatile organic substances has been demonstrated on the control of plant diseases. Today organic agriculture is an important problem of the worldwide and the hazards of excessive using of Fungicides is one of the concerns public health. Optimization and identification of effective bacterial volatile compounds can be reducing of chemical fungicides.

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