International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-7, Issue-1, 2016, pp248-254
http://www.bipublication.com

Research Article

Biological Control of Plant Fungal Diseases Using Volatile Substances of *Streptomyces griseus*

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[Received-21/02/2016, Accepted-27/02/2016, Published-06/03/2016]

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 Aspergillus niger, A.flavus, Fusarium oxysporum 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 tested fungi cultures were significantly suppressed in the presence of the volatiles. Gas chromatography comb with mass spectrometry analysis results showed that twenty volatile compound s 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 three abundant compound was analyzed. The volatile substances of S.griseus have a potential for using as a biofumigant to control plant fungal diseases.

Key words: Streptomyces griseus, Volatile substances, antifungal activity, DMSO, Carvacrol.

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 (Fravel 2005; Watve et al. 2001) and enzyme inhibitors (Singh et al. 1999). Bacterial volatile substances are among the secondary metabolites which have been recently investigated (Fernandoa et al. 2005; Kai et al. 2007) .Volatile substances produced by bacteria are substances with molecular weight of <300 Da, low polarity, and high vapor pressure (Pichersky et al. 2006). 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(Schöller et al. 2002) . Volatile substances derived from *Streptomyces sp*. and other species of actinomycetes prevent mycelium growth and inhibit spore germination of different fungi (Kai et al. 2007; Kai et al. 2008). Cyclohexanol, decanol,

2-ethyl-1-hexanol, nonanol, benzothiazole and dimethyl trisulfide are important compounds that inhibit spore germination and mycelium growth of *Sclerotinia sclerotiorum* (Fernandoa et al. 2005) . 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(Anitha and Rabeeth 2009; Anitha and Rabeeth 2010) **.** 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 (McCain 1966; Wan et al. 2008) . 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 (Li et al.2010) . 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 mycelium growth and spore germination of *A.niger*, *A.flavus,F.oxysporum* and *B.cinerea* and finally volatile substances of *S.griseus* were collected and analyzed. Fungistatic effect of some commercial volatile substance that produce by *S.griseus* cultures was studied.

Compound	Source	Purity
Carvacrol	SigmaAldrich	≥ 98%
DMSO	Sigma.Aldrich	≥ 99%
2,6-xyacetophenone	SigmaAldrich	≥ 97%

Table1. Abundant volatile substances from S. griseus cultures and analyzed inhibitory effect on mycelium tested fungi.

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 (HIMEDIA (ISP Medium No. 2), Peptic digest of animal tissue5gr/l, Yeast extract3gr/l, Malt extract3gr/l, Dextrose10gr/l, Agar20gr/l, pH: 6.2) 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. *A.niger*(PTCC 5154) , *A.flavus*(PTCC 5004) , *F.oxysporum*(PTCC 5115) were 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(HIMEDIA,M137, Malt extract 30gr/l, Mycological peptone 5gr/l, Agar15gr/l,pH:5.4) Fungi were incubated at 27 $^{\circ}$C for seven days. Stock cultures of each isolated species were maintained on Potato Dextrose Agar (PDA) (HIMEDIA, M096, Potato Infusion from (200 g) 4gr, Dextrose20gr, Agar15gr, pH:5.6) at 4°C.

Preparation of spore suspension

To prepare spore suspension, 20 ml of 5% (v/v) of Tween 80(Merck) was added to MEA culture media. Fungi cultures 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 tested fungi two bottom dishes of sterilized petri dishe (9cm in diameter) were used. In this method , *S.griseus* was superficially cultured on Tryptic Soy Agar (TSA) media (Merck, Peptone from casein 15gr/l, peptone from soymeal 5gr/l, sodium chloride 5gr/l, agar-agar 15gr/l, pH:7.3) in 9 cm plates by swap, the other plate containing MEA

inoculated with a 5mm diameter plug of 7day old fungi. The two dishes were sealed together by parafilm, to obtain a double dish chamber (Arrebola et al., 2010). All samples were inoculated for five 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 fungi, the inverse double technique were used as explained above. 20 µl of spore suspension (10^5 spore/ml) of fungi were inoculated on water agar (Agar 15gr/l) petri plates. 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 control. There were four replicates for each treatment and the experiments were repeated twice.

Collection and GC-MS analysis

On the filter paper. 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) (Merck) 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 °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.

Antifungal activity of volatile compounds

Commerical products of volatile substances which were most abundant components of *S.griseus* were purchased from Sigma).

Their fungistatic activities were assessed using perivious method.The top dish contained a peice of autoclaved filter paper (ca. 15mm × 15mm) and 150 l of the commercial volatile compounds was added

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 mycelium growth

The results showed that, the radial mycelial growth of fungi were significantly decreased by the volatiles from TSA cultures of *S.griseus* as compared with the control (without inoculation of *S.griseus*) .The most inhibitory effect were measured 8.1 mm for *F.oxysporum* and minimum inhibition was measured for *A.niger*(14.4mm) . (Table2.)

Table: 2.effect of the volatile substances produced by S.griseus on mycelium growth of pathogen fungi.

Fungus	Mycelium growth[a](mm)	
	Control	
A.flavus	43.70a	10.5b
F.oxysporum	41.3a	8.1b
A.niger	75.8a	14.4b
B.cinerea	42.1a	11b

[a] Mycelium growth were measured after incubation for 5 d at 30^0C.Means based 32 replicates followed by the same letters within the line were not significantly different($P<0.05$) according to the least Significant Difference test.

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

Spore germination of fungi were evaluated after 24 h incubation at 30 °C. No spore was germinated in treated samples, exept *A.flavus*. Maximum inhibition of spore germination by the volatiles of *S.griseus* for *F.oxysporum* was calculated 100%. In the controls plates, all the spores were germinated and colony formation was observed after 72h.Minimum inhibitory effect of germination was observed for *A.flavus* (56%) after 72h.(Table3.)

Table: 3. Inhibitory effect of spore germination by produced S.griseus

Fungus	Spore germination		
	24h	48h	72h
A.flavus	%85	%62	%56
F.oxysporum	%100	%100	%100
A.niger	%100	%98	%89
B.cinerea	%100	%90	%83

[a] Spore germination were measured after incubation for 3 d.Inhibition of germination were calculated using the formula: germination= (germination in the negative control-germination in the treatment) /(germination in the negative control) ×100%

GC-MS analysis of the volatiles of S.griseus

Analysis of GC-MS identified twenty volatile compounds released from diphasic culture of *S.griseus* 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 (Table4.) . Chemicals of less abundance were Isocyclocitral, Benzene,1,2-dimethoxy -4-1-methyletheny

Table4. GC-MS analysis of volatile compounds produced by S.griseus. RT: retention time.

RT[a](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	Malonic acid	73
20.99	0.50	161	1H-Cyclopropal [a] naphthalene	161
23.30	0.62	189	1,2,3,4,4a,5,6,8a-octahydro-4a,8-imethyl-2(1-methylethenyl	189
23.68	0.45	178	Benzene, 1,2-dimethoxy-4-(1-propenyl)	178
23.99	0.9	150	Cyclohexanone	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	1,3-benzodioxole	192.10
26.62	0.96	192	Dehydroxy-isocalamendiol	192
27.18	0.32	147.10	Cyclohexan	147.10
27.42	3.44	73	Ethanedioic acid	73
27.66	0.44	109	1,4-CIS-1,7-CIS-ACORENONE	109
29.61	1.40	73	SILICONE POLYMER	73
31.48	6.18	182	Trans-1,10-Dymethyl-trans-9-decalol	182
33.397	0.63	73	Silane 1,6-heptadiyne-1,7-diylbis (trimethyl)	73

Inhibitory effect of commercial volatile compounds on mycelium growth

Among the three volatile compounds tested,Carvacrol and DMSO completely inhibited mycelial growth of *F.oysporum, B.cinerea*.and *A.niger* so that didn't show mycelium growth after 5 days but midding inhibitory effect against *A.flavus*.2,6-dihydroxyacetophenone didn't have any inhibitory activity on mycelium growth of tested fungi.(Table5.)

Table: 5.Effect of commercial voltile compounds on control of mycelium growth of pathogenic fungi after 5d incubation.

Compounds	**Mycelium growth(mm) a**			
	A.flavus	*F.oxysporum*	*A.niger*	*B.cinerea*
Carvacrol	0	0	0	0
DMSO	21.4a	0	14.3b	0
2,6-dihydroxyacetophenone	NIb	NI	NI	NI

[a] Mycelium growth were measured after incubation for 5 d at 30^{0}C. Means followed by the same letters within the line were not significantly different($P<0.05$) according to the least Significant Difference test.

[b] NI,not inhibition with c

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 *A.flavus,F.oxysporum.A.niger* 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* (Figiela et al. 2010) . The cytotoxic ability of Carvacrol on peroxidant activity can make it an effective antiseptic and antimicrobial agent (Bakkali et al. 2008) . Previous investigations showed that Carvacrol has antifungal activities against *Penicillium glabrum, P. capisci, R.solani, F.moniiliforme, S.sclerotiorum* and *Cladosporium herbarum* (Andersen 2006).

In this study demonstrated carvacrol inhibited of mucelium growth on tested fungi compeletly. Another volatile substance detected in this study was Dimethyl sulfoxide (DMSO) which is used as a routine solvent for antifungal drugs (Randhawa 2006). Antifungal activity of DMSO demonstrated by different studies (Jessup et al.2000; Voda et al. 2004) . Elad showed DMSO reduces linear growth

of *Botrytis cinerea* isolates, which cause gray mold in potato(Elad 1992) .Results showed DMSO suppression of mycelium growth on *B.cinerea* and *F.oxysporum* completely .Significant differences between the volatiles compounds of *S.griseus* and some other Actinomycetes (Dickschat et al. 2001; Wan et al. 2008) were also found. Thus, different species may release similar and dissimilar types of volatile compounds, with various effects(Li et al. 2010) . Different bacterial and fungal diseases which may occur at harvest, storage and transport (Eckert and Ogawa 1988) 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 (Adaskaveg et al. 2002) . 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 (Mercier and Jimenez 2004) and in greenhouses (Koitabashi 2005) . The Effect of volatile substances of Bacillus pumillus (Fernandoa et al. 2005) ,B.subtilis (Freire et al. 2012) and S. platensis F1 (Wan et al. 2008) showed that these compounds inhibited mycelium growth and spore germination of *B.cinerea*. The demonstrated effects of volatile substances of *S.griseus*, (inhibition of spore germination and mycelium growth) have a potential for the effective control of *A.flavus,F.oxysporum,A.niger* and *B.cinerea*. Jain et al.,showed the fungitoxic effect of seven volatile substances (ammonia, carbon disulphide, petroleum benzene, carbon dioxide, methanol, glacial acetic acid and hydrogen peroxide) against *A.niger, A.flavus*. In the other study Özer et al(2009) demonstrated Volatile compounds in twelve of the soil samples strongly (>70%) inhibited spore germination of *A.niger*. Moore-Landecker and Stotzky (1972) showed that microbial volatile could inhibit mycelium growth, spore formation, alternation of colony and morphology of *F.oxysporum.* 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 *A.flavus, F.oxysporum,A.niger* and B.cinerea *in vitro*. In our study, microscopy picture showed that spores exposed to the volatiles did not germinate after 24 h, even after washing.In conclusion, the present study showed that the volatiles from *S.griseus* and their components affect plant pathogenic 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 all individual compounds and mixtures on plant diseases.

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