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Identification of some secondary metabolites produced by four *Penicillium* species

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Abstract: Fungi produce a wide range of secondary metabolites such as antibiotics, toxins, alkaloids, fatty acids, ketones and alcohols during active cell growth. The present study aimed to identify the secondary metabolites from some *Penicillium* species, using GC-MS. Many important compounds such as 3-oxoquinuclidine in *Penicillium jenseii*, formamidine in *Penicillium pusillum*, orcinol and 1,3,8-p-menthatriene in *Penicillium canescens* and limonene in *Penicillium purpurogenum* were identified. Moreover, fatty acids and hydrocarbons were produced by all the tested species.

Key words: biological compounds, *Penicillium*, GC-MS

INTRODUCTION

Fungi produce primary metabolites (amino acids, proteins, carbohydrates, vitamins, acetone, ethanol, etc.) and secondary metabolites (antibiotics, toxins, alkaloids, fatty acids, ketones, alcohols, etc.) during active cell growth (Devi et al. 2009). However, these components play no role in primary metabolism of fungi. Hawksworth (2001) studied fungal biodiversity and suggested that nearly 1.5 million fungal species exist on earth, from which just 5% are identified so far. Soil fungi are substantially prolific sources of highly bioactive secondary metabolites, which is interesting as a complex aspect of fungal development (Bok & Keller 2004). Identification of a number of new metabolites and evaluation of their biological activities have been studied (Gao et al. 2010).

Penicillium is an anamorphic ascomycete and comprises more than 200 species, many of which are common soil inhabitants. *Penicillium* species are important because of their widespread occurrence and ability to produce a wide range of bioactive secondary metabolites, including antibacterial, antifungal, immune suppressants, cholesterol-lowering agents and mycotoxins (Petit et al. 2009).

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The fungi producing secondary metabolites have industrial application. Thus, identification of these components and optimization of the fungal growth conditions can help us manage optimum production of secondary metabolites. In this study, we aimed to identify some secondary metabolites produced by four *Penicillium* species, including *P. jenseii*, *P. pusillum*, *P. purpurogenum* and *P. canescens*, using gas chromatography combined with mass spectrometry (GC-MS). The top biological activities of major metabolites were described according to the related references.

MATERIALS AND METHODS

Growth conditions

Penicillium species were prepared of living cultures from laboratory of mycology, department of plant pathology at the Sari Agricultural Sciences and Natural Resources University. Then, spores were suspended in potato dextrose broth (PDB) and incubated at 25°C on a shaker for 14 days at 130 rpm (Siddiquee *et al.* 2012).

Extraction procedure

The metabolites were determined using gas chromatography according to the method of Siddiquee et al. (2012), with some modifications. Extraction was performed by adding 10 ml Ethyl acetate ($C_4H_8O_2$) to 50 ml liquid culture in an Erlenmeyer flask, and the mixture was incubated at 4°C for 10 min. Then, flasks were shaken for 20 min at 130 rpm. The supernatant (metabolites and $C_4H_8O_2$) was separated from the liquid culture and evaporated, using a rotary evaporator at 45°C. The residue was dissolved in one ml methanol (CH_4OH), filtered through a 0.2 µm syringe filter (Millipore) and stored at 4°C for 24h before injection to GC-MS.

GC-MS conditions

An Agilent technologies 7890 A gas chromatograph connected to a 5975 Cinert MSD was used for identification of secondary metabolites from the crude extract of *Penicillium* species. In addition, an HP-5MS fused silica capillary column (Hewlett-Packard, 30 m* 0.25 mm i.d., 0.25 µm film, cross-linked to 5% phenyl methyl siloxane stationary phase) was applied. The entire system was controlled by Chemstation software (Hewlett-Packard, version

A.01.01). Electron impact mass spectra were recorded at 70 electron voltage and ultra-high pure He (99.999%) gas was used as the carrier gas at flow rate of 1 mL/min. The injection volume was 1 µL and all the injections were performed in a split-less mode. Temperature of the injector and detector was 250 and 280°C, respectively. Column oven temperature was initially set at 50°C for 5 min, then increased to 260°C (ramp: 4°C/min) and held for 5 min.

RESULTS AND DISCUSSION

The retention time and abundance of the compounds under the described conditions in GC-MS section are shown in tables 1, 2, 3 and 4.

In this study, various secondary metabolites were detected within *Penicillium* species. We have described the biological activity of certain metabolites and compared the species based on the produced metabolites.

Many of the important compounds were produced by *Penicillium jenseii*. Some of them were not detected in any of the other species, for instance 3-oxoquinuclidine. This compound is a derivative of quinuclidine (1-azabicyclo [2.2.2] octane), which has a wide variety of biological activities and has a substantial importance as a structural fragment of a synthetic pharmaceutical compound (Odzak et al. 2007).

Quinoline is an alkaloid mycotoxin (Kozlovsky 1990). Qunnoline and isoquinoline compounds have antiprotozoal activity, so that malaria infections are treated by several drugs, including quinoline (Osorio et al. 2008). Quinoline and 5-phenylisoquinoline were detected only in *P. pusillum*. Moreover, formamidine and uvidin B were detected in *P. pusillum*, but not in the other *Penicillium* species. There have already been many reports concerning the various biological activities and properties of formamidines, including acaricidal, bactericidal, antiprotozoal, antihelminthic, fungicidal and herbicidal effects (Hollingworth 1976). Uvidin B which is one of the drimane-type sesquiterpenes, was isolated from *Lactarius uvidus* by Bernardi et al. (1980).

Limonene, the terpenoid compound was detected only in *P. purpurogenum*. This compound was reported to be the major component in the extract prepared from cultivated *Trichoderma* sp. (Khethr *et al*. 2008). They also investigated the antibacterial and antifungal activities of limonene against five pathogenic gram positive and gram negative bacterial strains and five pathogenic fungi, and reported that the compounds have positive antibacterial, but no antifungal activity. Limonene has been reported as one of the secondary metabolites in *P. olsonii*, *P. roqueforti* and *P. vulpinum* (Frisvad et al. 2004).

Thymol is an essential oil with strong antifungal activity (Sokovic et al. 2009). Previous investigations by Sokovic & Griensven (2006) show that thymol has a very high activity against three major pathogens of the button mushroom, *Agaricus bisporus*, including *Verticillium fungicola* , *Trichoderma harzianum* and *Pseudomonas tolaasii*. This compound has also showed a very strong antibacterial activity against food spoilage bacteria (Sokovic et al. 2007) and was detected only in *P. purpurogenum* in the present study.

Table 1. Compounds produced by *Penicillium pusillum* identified by GC-MS.

Compounds	R_T, min	Abundance(%)	Compounds	R_T, min	Abundance(%)
Decanedioic acid	27.916	14	Uvidin B	62.13	81
Hexadecane	35.143	38	Tridecane	21.095	90
Decane	7.574	43	Camazulene	37.403	90
Azetidine	28.660	43	5-Phenylisoquinoline	32.018	93
N-formylpiperidine	28.396	46	Dodecane	16.615	95
Isolongifolen-5-one	38.496	46	Tetradecane	25.369	96
Quinoline	29.850	47	Dodecanoic acid	33.266	96
Formamidine	35.961	70	Dibenzothiophene	38.038	96

Table 2. Compounds produced by *Penicillium jenseii* identified by GC-MS.

Compounds	R_T, min	Abundance(%)	Compounds	R_T, min	Abundance(%)
Sarcocapnidine	19.060	7	Thioxanthene	37.771	86
Pyrimidin-4-one	29.869	7	Hexadecane	38.234	87
Stannane	34.389	7	Decane	9.825	91
Octadecanoic acid	22.236	9	Undecane	13.864	91
Neoisolongifolene	35.402	10	Anthracene	38.343	93
4-Fluoroveratrole	24.725	25	3-Oxoquinuclidine	34.062	95
1-ethylisatine	37.605	35	Eicosane	40.615	95
Methylstilbene	35.957	38	Tetradecane	24.358	96
Oxalic acid	24.107	39	p-Xylene	5.682	97
Adamantanecarboxanilide	47.229	50	2,4-Nonadiyne	9.470	97
Phenanthrene	34.830	53	Dodecane	17.601	97
Dibenzothiophene	34.149	83	Hexadecanoic acid	38.881	97

Unique metabolites detected in *P. canescens* include orcinol, 1,3,8-p-menthatriene and aphthosin. Orcinol is a member of fumigatins family, one of the secondary metabolites (Frisvad et al. 2008). Fumigatins were reported as active antibiotics against some gram-negative and gram-positive bacteria (Waksman & Geiger 1944). Frisvad et al. (2008) reported the production of at least 226 potentially bioactive secondary metabolites by *Aspergillus fumigatus*, including orcinol.

Taran et al. (2010) demonstrated the antimicrobial activity of 1, 3, 8-p-menthatriene and some other essential oils of *Ferulago angulata* subsp. *carduchorum*. *Staphylococcus aureus* (MIC=15µg/ml) and *Listeria monocytogenesis* (MIC=137µg/ml) showed a high sensitivity to essential oils of aerial parts and seeds, respectively. *Ferulago* species have sedative, tonic, digestive and antiparasitic effects (Baser et al. 2002). Aphthosin, the first example of tetradepsides was isolated from *Peltigera aphtosa* by Bachelor & King (1970).

Phenanthrenes are from a rather uncommon class of aromatic metabolites. A large number of different phenanthrenes has been reported to occur in plants and studied for their cytotoxicity, phytotoxicity, antimicrobial, spasmolytic, anti-inflammatory, antiplatelet aggregation and antiallergic activities (Kovacs *et al.* 2008). Phenanthrene was produced by all the *Penicillium* species in this study, except *P. pusillum.*

Anthracene is a polycyclic aromatic hydrocarbon, consisting of three fused benzene rings. Krivobok et al. (1998) studied the toxicity of anthracene on 39 micromycetes. There are also some other reports demonstrating that white rot fungi (Bezalel et al. 1996) and some other fungi, including *Cunninghamella elegans* and *Rhizoctonia solani* (Sutherland 1992) are able to metabolize anthracene. In this study, we found anthracene in the culture of *P. jenseii*, *P. purpurogenum* and 9,10-anthracenedione in *P. canescens.* Also, thioxanthene was produced by all the species, except *P. pusillum.* Kristiansen and Vergmann (1986) investigated the antibacterial effect of various thioxanthene derivatives on mycobacteria *in vitro* and mentioned that the antibacterial capacity against the slow-growing mycobacteria is the same as stereo-isomeric analogs of thioxanthene derivatives. Ford et al. (1990) studied the structure-activity relationship of a series of thioxanthene isomers in a multidrug resistant (MDR) human breast cancer cell line.

Sarcocapnidine, the isocularine alkaloid was produced by *P. purpurogenum* and *P. jenseii.* This compound was reported to be the major alkaloids component in the genus *Sarcocapnos* by Suau *et al*. (2005).

Dibenzothiophene (DBT) is the organic sulphur that was produced by *P. purpurogenum, P. jenseii* and *P. pusillum.* Many of the bacteria, such as *Brevibacterium* and *Pseudomonas* utilize DBT as the source of carbon, sulfur and energy (Acharya et al. 2005).

Table 3. Compounds produced by *Penicillium purpurogenum* identified by GC-MS.

Compounds	R_T, min	Abundance(%)	Compounds	R_T, min	Abundance(%)
Azetidine	23.609	4	Decane	9.825	91
Thymol	20.914	5	Dibenzothiophene	36.718	93
Sarcocapnidine	19.071	7	Anthracene	34.830	93
Octadecanoic acid	22.236	9	Tetradecane	24.358	96
2-(2-Methylvinyl)thiophene	23.670	38	Heptadecane	38.228	96
Methoxyacetic acid	29.508	43	Phenanthrene	38.337	96
Nonadecane	27.444	59	p-Xylene	5.648	97
Camazulene	32.627	70	Dodecane	7.595	97
Limonene	10.952	87	Hexadecane	30.334	97
Thioxanthene	37.771	90	Octadecane	35.722	98

Table 4. Compounds produced by *Penicillium canescens* identified by GC-MS.

Compounds	R_T, min	Abundance(%)	Compounds	R_T, min	Abundance(%)
1,3,8-p-Menthatriene	13.166	9	Thioxanthene	37.771	90
Orcinol	28.118	27	Decane	9.825	91
Nicodicodine	60.614	38	Eicosane	40.615	93
Borane	16.508	40	di-p-Tolylacetylene	41.324	93
Adamantanecarboxanilide	17.595	43	Tetradecane	24.358	96
Aphthosin	32.466	47	Hexadecane	30.334	96
Diethyl Phthalate	30.189	59	Heptadecane	38.288	96
9,10-Anthracenedione	40.168	59	p-Xylene	5.796	97
Phenanthrene	34.830	70	Dodecane	17.595	97
Oxalic acid	20.061	74	Hexadecanoic acid	38.881	98
Tridecane	23.403	83	-	-	

There have been several reports about the production of isolongifolene derivatives in the fungi and plants. Hang et al. (2012) analyzed the isolongifolen-5-one and other volatile components from *Dictyophora rubrovolota*. This compound was found in *P. pusillum* in this study. Neoisolongifolene reported as one of the essential oils of the eaglewood tree (*Aquilaria agallocha*) (Bhuiyan et al. 2008), was detected in *P. jenseii* in the present study.

Oxalic acid is a secondary metabolite produced by several organisms. It can be found extensively in nature and secreted to the environment by many fungi. Production of oxalic acid is beneficial to the producing organisms, to some extent (Blumenthal 2004). Cessna et al. (2000) reported that oxalic acid seems to be the pathogenicity factor in *Sclerotinia sclerotiorum.* Dutton and Evans (1996) studied the production of oxalic acid in fungi. They expressed that it affects the pathogenicity through acidification of the host tissues and causes the sequestration of calcium from the host cell. Jarosz-Wilkolazka and Gadd (2003) reported that the wood-rotting fungi produce oxalic acid as a major metabolite in response to toxic stress. In the present study, this compound was produced by *P. canescens* and *P. jenseii.*

Camazulene and Azetidine were produced by *P. pusillum and P. purpurogenum.* Camazulene is an essential oil with antifungal activity (Liu et al. 2001). Azetidine is an alkaloid compound possessing potent actomyocin ATP ase-activating properties (Pinder 1992).

Commonly, the fatty acids and hydrocarbons are produced by *Penicillium* species. Fatty acids are organic acids with antibacterial, antimalarial and antifungal activities (Pohl et al. 2011). Fatty acids are present anywhere in nature and are also physiologically important class of molecules involved in cell energy storage, membrane structure and in various signaling pathways (Liu et al. 2008). Pohl et al. (2011) reported that fatty acids and their derivatives are very important as antifungal agents and also are used as novel antifungal compounds. They concluded that the most important target of antifungal fatty acids is the cell membrane, and reported that augmentation of the membrane fluidity is partly because of the presence of fatty acids, which results in discharge of the intracellular components and cell death. Similarly, Helander et al. (1998) reported that the outer membrane disintegrating properties may be the cause of the action of the oil. Some studies suggest that these compounds permeate inside the cell, where they are inhibited by cellular metabolism (Marino et al. 2001). Other studies explain the deterioration of cellular membrane structure and reaction of the active sites of enzymes or their act as a H+ carrier, exhausting the adenosine triphosphate (ATP) pool (Farag et al. 1989). Dodecanoic (lauric) acid, as an unsaturated fatty acid was produced by *P. pusillum.* On the other hand, the fatty acids reported by Pohl et al. (2011), including hexadecanoic (palmitic) acid, octadecanoic (stearic) acid and decanedioic acid were produced by *P. canescens* and *P. jenseii*, *P. jenseii* and *P. purpurogenum*, and *P. pusillum*, respectively.

The hydrocarbons, such as decane, dodecane, tridecane, heptadecane, eicosane, hexadecane and undecane were produced by all the tested *Penicillium* species.

Some secondary metabolites are involved in the complex interactions between fungi and their living environment. Thus, analytical methods for the identification of compounds are useful to study their functions in biological interactions. The GC–MS technique is a powerful tool for the study of the dynamic range of secondary metabolites. Based on the specific criteria like mass-spectral factor, the application of this method shows a wide variety of major metabolites in *Penicillium* species. According to the trait of the compounds described in this study, we suggest the usage of complementary methods for purification of *Penicillium* species compounds, due to their application in agriculture as biological control agents of plant pathogens, or in medicine as sources of antimicrobial substances.

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شناسایی برخی متابولیت های ثانویه درچهار گونه ی *Penicillium*

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چکیده: قارچ ها شمار زیادی متابولیت ثانویه از جمله آنتی بیوتیک ها، توکسین ها، آلکالوئیدها، اسیدهای چرب، کتون ها و الکل ها را در طی رشد سلولی تولید می کنند. مطالعه حاضر به منظور شناسایی طیف وسیعی از متابولیت های ثانویه در برخی گونه های *Penicillium* با استفاده از روش GC-MS انجام شده است. بسیاری از ترکیبات مهم مانند 3-oxoquinuclidine در *Penicillium jenseii*، formamidine در *Penicillium pusillum*، orcinol و 1,3,8-p-menthatriene در *Penicillium canescens* و limonene در *Penicillium purpurogenum* شناسایی شدند. علاوه بر این، اسیدهای چرب و هیدروکربن ها در تمام گونه های بررسی شده تولید شده بودند.

کلمات کلیدی: ترکیبات بیولوژیکی، *Penicillium*، GC-MS.

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