Journal of Microbiological Methods 90 (2012) 327–335

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Mass spectrometry-based chemotaxonomic classification of *Penicillium* species (*P. echinulatum, P. expansum, P. solitum*, and *P. oxalicum*) and its correlation with antioxidant activity

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ARTICLE INFO

Article history:
Received 23 March 2012
Received in revised form 11 June 2012
Accepted 13 June 2012
Available online 23 June 2012

Keywords:
Antioxidant activity
Chemotaxonomy
GC-IT-MS
LC-ESI-MS
Penicillium
PLS-DA

ABSTRACT

In this study, 4 *Penicillium* species (17 strains) were classified on the basis of metabolite profile (chemotaxonomy) by using liquid chromatography-electrospray ionization ion trap-mass spectrometry (LC-ESI-MS), gas chromatography-ion trap-mass spectrometry (GC-IT-MS) and multivariate statistical analysis. The LC-ESI-MS-based dendrogram was similar to the internal transcribed spacer (ITS)-based dendrogram, in that *Penicillium oxalicum* was separated from the other 3 species. Moreover, vermiculidiol, meleagrin, oxaline, glandicolin A and B, and secalonic acid D were identified as metabolites that enable discrimination of *Penicillium* species by partial least squares discriminant analysis (PLS-DA). Evaluation of the species-specific metabolites produced by *P. expansum*, *P. echinulatum*, and *P. solitum* revealed that the 3 species differed from each other. On the other hand, GC-IT-MS-based dendrogram revealed that *P. expansum* was clearly classified separately from the other 3 species, and this result correlated with the antioxidant activity of the 4 species: *P. expansum* had a higher radical scavenging activity than the other 3 species. The metabolites produced in higher amounts in *P. expansum* were gluconic acid (12, 29, 33); andrastin A (16), B (15), and C (17); chaetoglobosin C (14), a class of sugar (31, 32); and salicylic acid (28). The results of this study demonstrated that metabolite-based chemotaxonomy could be used not only as a classification method but also as a tool for evaluation of species-specific activities.

1. Introduction

Penicillium is one of the well-known groups of fungi, containing over 200 species with a worldwide distribution. In Korea, about 60 species of *Penicillium* have been found, and 10 of these were reported to be associated with storage diseases of plants. The major *Penicillium* species derived from stored fruits are *P. echinulatum, P. expansum, P. oxalicum*, and *P. solitum* and they cause damage to many fruits, such as onions, apples, pears and citrus fruits (Kim et al., 2002). However, it is not easy to classify *Penicillium* species, because of the large variability within the species; therefore, a system that enables unambiguous identification of these species is required. In addition, some plant-derived *Penicillium* species not only damage plants but can also harm animals by producing metabolites, such as patulin and citrinin, which are mycotoxins. The potential of a particular species of *Penicillium* to cause harm to animals makes it very important to clearly classify and differentiate the various species and identify species-specific metabolites. Traditional taxonomic classification of *Pencillium* is difficult, since this method of classification relies on information such as teleomorphic states and morphological criteria, which are not clearly defined for *Penicillium*. To classify the *Penicillium* species, different methods have been used, such as metabolite profiling (chemotaxonomy), DNA fingerprinting, and physiological and morphological analyses. Among these methods, chemotaxonomy, a metabolite-based classification method, has shown great value in the differentiation of Ascomycota (Hettick et al., 2008). It is useful for identification of closely related *Penicillium* species. Frisvad and Filtenborg (1983) reported the classification of terverticillate penicillia based on mycotoxins and secondary metabolite profiling. And, Smedsgaard and Frisvad (1997) demonstrated that 58 *Penicillium* species were classified and some species-specific metabolites were recommended *via* the metabolite-based profiling of terverticillate penicillia by using electrospray mass spectrometry. Even though many authors have indicated that metabolites may be correlated with taxonomic differentiation, the correlation between the metabolite profile and taxa in the *Penicillium* species is poorly characterized, especially for the plant-derived *Penicillium* species in Korea. Moreover, *Penicillium* species are well known for producing metabolites with antioxidant activity. Atrovenetin (Ishikawa et al., 1991), 2,3-dihydroxybenzoic acid (Hayashi et al., 1995), and gentisyl alcohol (Alfaro et al., 2003) from various *Penicillium* species are such examples. In this study, we analyzed metabolites of plant-derived *Penicillium* species by

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doi:10.1016/j.mimet.2012.06.006

using liquid/gas chromatography–electrospray ionization iontrap mass spectrometry (LC-ESI-MS/GC-IT-MS) and multivariate statistical analysis to understand how the metabolites influence taxonomy. In addition, we evaluated the antioxidant activity of *Penicillium* species extracts to determine how metabolites and their species-specific variation affect the antioxidant activity.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade water, methanol, and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA). Malt extract agar (MEA) was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Analytical-grade formic acid, pyridine, methoxyamine hydrochloride and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Fungal strains and culture conditions

The *Penicillium* strains were obtained from the Korean Agricultural Culture Collection (KACC, South Korea). All cultures were maintained at 28 °C on MEA. All *Penicillium* strains were cultured on MEA plates for 3 days and pieces of young mycelia (6 mm in diameter) were placed on new MEA plates. Seventeen strains of *Penicillium* were extracted after 12 days of incubation, and the extracts from these strains were used in LC/GC-MS and UPLC-Q-TOF MS analyses.

2.3. Extraction of fungal metabolites

To prepare *Penicillium* extracts, 3 mL of ethyl acetate was added to four 6-mm agar pieces, which were then mixed using a rotary shaker at 200 rpm for 9 h (Nielsen et al., 2005). The extracts were evaporated to dryness, dissolved again in methanol and then passed through a disposable 0.45-μm polytetrafluoroethylene (PTFE) filter before injection into the LC-ESI-MS spectrometer. The extracts used in LC-ESI-MS analysis were evaporated and derivatized for GC-IT-MS analysis. The re-dried samples were dissolved in 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) and heated at 30 °C for 90 min. Subsequently, after 10 min, 100 μL of BSTFA was added, and the samples were incubated at 37 °C for 30 min. Finally, the products were filtered through a PTFE filter and used for GC-IT-MS analysis.

2.4. Analysis of Penicillium sp. Metabolites

2.4.1. LC-ESI-MS analysis

Liquid chromatographic analysis was performed on a Varian 500MS ion trap mass spectrometer (Varian Inc., Palo Alto, CA, USA), which consisted of an LC pump (Varian 212), a photodiode array detector (ProStar 335) and an auto sampler (ProStar 410). The LC system was equipped with a C_{18} column measuring 100 × 2.0 mm in size and a 3-μm particle size (Varian Inc., Palo Alto, CA, USA). The binary mobile phase consisted of water and acetonitrile with 0.1% formic acid (v/v). The initial condition of the mobile phase was 10% acetonitrile for 2 min, and the gradient was gradually increased to 100% acetonitrile over 28 min. The gradient was maintained at 100% acetonitrile for 5 min, and then acetonitrile was sharply reduced to 10% over 0.06 min and maintained at 10% for 5 min. Ten microliters of each sample was injected, and the flow rate was maintained at 0.2 mL/min. ESI-MS was performed in a negative mode within a 100–1000 *m/z* range. The running parameters were as follows: drying temperature, 350 °C; needle voltage, 5 kV; capillary voltage, 70 V; drying gas pressure (nitrogen) 10 psi, and nebulizer gas pressure (air) 35 psi. MS^n analysis was performed using scan-type turbo data-dependent scanning (DDS) at the same conditions used for the negative- and positive-mode MS scanning.

2.4.2. GC-IT-MS analysis

The GC-MS analyses were performed with a Varian 4000 ion trap mass spectrometer (Varian Inc., Palo Alto, CA). The column was 30 m × 0.25 mm and had a particle size of 0.25 μm (Varian Inc., Palo Alto, CA, USA). The temperatures of the injector and the ion source were 250 °C and 200 °C, respectively. The flow rate of the helium gas was 1 mL/min, and the electron impact voltage was 70 kV. The column temperature was maintained at 70 °C for 2 min and then raised to 300 °C at 10 °C/min. The analysis range of mass was 50 to 1000 *m/z*.

2.4.3. Ultra performance liquid chromatography quadruple time-of-flight mass spectrometry (UPLC-Q-TOF-MS) analysis

To accurately measure the molecular weight of the selected metabolites, UPLC-Q-TOF-MS analysis was performed using a Waters Micromass Q-TOF Premier with UPLC Acquity system™ (Waters, Milford, MA, USA). The UPLC system contained an Acquity UPLC BEH C_{18} column that was 100 × 2.1 mm and had a particle size of 1.7 μm (Waters, Milford, MA, USA). The mobile phase was a modified version of that used for the LC-ESI-MS system. The initial condition of the mobile phase was 0% acetonitrile for 0.3 min, and the gradient was gradually increased to 30% acetonitrile over 3 min, and then increased to 40% acetonitrile for 1 min, followed by an increase to 100% acetonitrile for 8 min. The mobile phase was held at 100% acetonitrile for 2 min, followed by 0% acetonitrile for 2 min. Five microliters of the sample was injected, and the flow rate was maintained at 0.3 mL/min. Electron spray ionization was performed in the negative and positive mode within a 100–1000 range of *m/z*. The operating parameters were as follows: ion source temperature, 200 °C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; capillary voltage, 2.8 kV; and cone voltage, up to 35 V.

2.5. Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) free radical scavenging activity of extracts of Penicillium strains

The free radical scavenging activity of each extracts from 17 *Penicillium* strains was measured using a protocol described previously by Re et al. (1999). To determine the ABTS free radical scavenging activity, 7 mM ABTS (ammonium salt) was dissolved in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl, and treated with 2.45 mM potassium persulfate. The concentration of the resulting blue-green ABTS radical solution was calibrated to an absorbance of 0.650 ± 0.020 (mean ± SD) at 734 nm. After 20 μL of each crude extract solution had been added to 180 μL of the ABTS radical solution, the samples were incubated in darkness at 37 °C for 7 min, and the decrease in absorbance at 734 nm was measured using a microplate reader (Bio-Tek Instruments). The control sample contained 20 μL of methanol and 180 μL of the ABTS solution. The results were expressed as a percentage of radical scavenging activity of methanolic extracts from *Penicillium* strains. Statistically significant differences ($p<0.05$) were evaluated using Duncan's multiple-range test. All experiments were carried out in triplicate.

2.6. Data processing

The LC-ESI-MS data were analyzed using Varian MS Workstation 6.9 software (Varian Inc., Palo Alto, CA, USA). The LC-ESI-MS negative-mode chromatogram raw data files were converted into the network common data form (netCDF, *.cdf) by using the Vx Capture software 2.1 (Adron Systems LLC, Laporte, MN, USA). The netCDF files were automatically aligned and compared with the mass spectrometry datasets by using the MetAlign software package (http://www.metalign.nl) (Lommen, 2009). The MetAlign parameters were set as follows: peak slope factor of 1.0, peak threshold factor of 2.0, peak threshold of 15 and average peak width at half height of 7.0,