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Indole-3-Acetic Acid Production by *Colletotrichum siamense*, An Endophytic Fungus from *Piper nigrum* Leaves

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Abstract: Plant endophytic fungi have been recognized as an important and a good source of natural bioactive products with potential application in agriculture, medicine and food industry. Chemical and biological studies of fungal metabolites originating from endophytic fungi isolated in Sri Lanka are relatively less studied. Therefore we investigated the chemistry and bioactivity of endophytic fungi present in leaves of *Piper nigrum* L. (black pepper) of the family Piperaceae famed as the spices king due to its pungent quality. A fungal endophyte isolated from the leaves of *P. nigrum* was identified as *Colletotrichum siamense* through molecular biological means using internal transcribed spacer (ITS) region of rDNA gene. This is the first report of the isolation of *C. siamense* from Piperaceae. The fungus was fermented in potato dextrose media and the fungal media were extracted with EtOAc. Chromatographic separation of the EtOAc extract over silica gel, Sephadex LH-20 and preparative thin layer chromatography furnished indole-3-acetic acid (IAA) and uracil. IAA showed high antifungal activity against the common plant pathogenic fungus *Cladosporium cladosporiodies* by TLC bioautography method and antioxidant activity against DPPH radical by spectrophotometry method.

Key words: Endophytic fungi; *Piper nigrum*; *Colletotrichum siamense*; Indole-3-acetic acid; Uracil.

Introduction

Microorganisms have been recognized as a promising source of novel bioactive compounds with potential application in agriculture, medicine and food industry. Penicillin, the first broad-spectrum antibiotic, is the most famous secondary metabolite originated from fungus *Penicillium notatum* [10]. Existing drugs of fungal origin include β-lactam antibiotics, griseofulvin, cyclosporine A, taxol, ergot alkaloids and lovastatins [23]. Endophytes, microorganisms that reside in the tissues of living plants, are recognized as a promising source of novel compounds. Some endophytic fungi have the ability to produce the same compounds that are produced by their host plant. Camptothecin, huperzine A, podophyllotoxin, taxol, vinblastine and vincristine are examples of such compounds [25]. We have previously reported several compounds with interesting bioactivities isolated from the endophytes isolated from Sri Lankan plants [3,15,16,18,19,21,24]. In a continuation of our studies on bioactive secondary metabolites produced by fungal endophytes associated with Sri Lankan plants, we investigated metabolites of an endophytic fungus, *Colletotrichum siamense* that was isolated from the leaves of the popular condiment plant *Piper nigrum* L. (black pepper) of the family Piperaceae. *P. nigrum* seeds are very popular

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spice used heavily in worldwide. Various parts of *P. nigrum* and its secondary metabolites are used as drugs, preservatives, and natural controlling agents. Its biological role and medicinal uses are described in a recent review [2]. In this paper we report the isolation of indole-3-acetic acid (**1**) and uracil (2) (Figure 1). Compound (**1**) showed antifungal activity against a common plant pathogenic fungus *Cladosporium cladosporioides* and antioxidant activity against DPPH (2,2-diphenyl-1-picrylhydrazyl).

Materials and methods

General

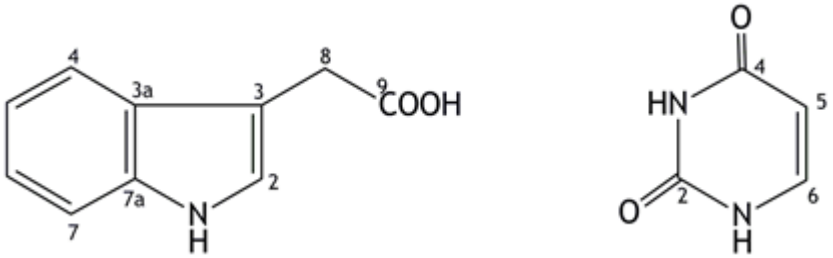
Extractions were taken using a sonicator (VWR Ultrasound cleaner, model-USC 1700 D). TLC analysis was conducted on silica gel plates (Merck 1.05554.0007, $60F_{254}$). TLC spots were located using a UV lamp and by heating after spraying with acidic anisaldehyde. Silica gel (Merck Art. 7734 & 9385) and Sephadex LH-20 were used for column chromatography. ^{1}H and ^{13}C NMR were recorded on a Bruker DRX500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) or a JEOL AL-300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer in CD_3OD solution. ^{1}H Chemical shifts are referenced to the residual proton signal of the solvent (δ 3.30), while ^{13}C chemical shifts are expressed in reference to the solvent signal (δ 49.0). FABMS were obtained on a JEOL JMS-700 spectrometer.

Isolation of endophytic fungus

Healthy and mature leaves of *P. nigrum* were collected from the Central Province of Sri Lanka in Feb, 2015. Leaves were washed gently with running water, and sterilized with 5 % NaOCl and distilled water three times. A segment of triple sterilized plant samples were placed on the surface of potato dextrose agar (PDA) medium and incubated at room temperature in darkness. After five days, emerging fungi were sub-cultured to obtain a pure culture of endophytic fungus which was identified as *Colletotrichum siamense* by molecular biological methods on the basis of the sequence of internal transcribed spacer (ITS) regions of the fungal rDNA gene, which was amplified using ITS1 ITS4 primers. BLAST search indicated that the sequence matched 99 % with those of *Colletotrichum siamense* strain C 1276.2 (GenBank accession No. JX010163.1). Photographic evidence of the leaves of *P. nigrum* and *C. siamense* strain (IFS/VPND) are deposited at the National Institute of Fundamental Studies.

Fermentation of the fungus and isolation of secondary metabolites

Large scale culturing of the fungus was carried out by inoculating *C. siamense* culture grown on PDA medium to Erlenmeyer flasks (1 L x 30), each containing 400 mL of potato dextrose broth (PDB), which allowed to stand 10 days and then incubated while shaking (95 rpm) every other day for another 18 days. The resulting culture broth was filtered and extracted with EtOAc. The mycelium was separately extracted with EtOAc using sonicator for three times. The two EtOAc extracts showed closely similar TLC pattern. Hence they were combined (2.58 g) and screened for antifungal activity against *C. cladosporioides* using TLC bioautography method [8], antioxidant activity using DPPH radical scavenging assay [26], brine shrimp toxicity [11], phytotoxicity in lettuce seeds germination [16] and α-amylase inhibitory activity [14]. The EtOAc extract was chromatographed



1 **2**

Figure 1. Structures of compounds **1** & **2**

over silica gel, Sephadex LH-20 and PTLC to furnished compound **1** (22 mg) and **2** (6 mg). Compound **1** was subjected to bioassay described above except for phytotoxicity.

Indole acetic acid (**1**): White leaflets (crystallized from $CHCl_3$); mp 162-163°C (decompose); ^{1}HNMR (500 MHz, CD_3OD): δ 7.53 (1H, *d*, 8.0, H-4), 7.31(1H, *d*, 8.0, H-7), 7.14 (1H, *s*, H-2), 7.08 (1H, *t*, 8.1, H-6), 6.99 (1H, *t*, 7.9, H-5), 3.72 (2H, *s*, H-8); ^{13}CNMR (125 MHz, CD_3OD): δ 119.4 (C-2), 128.7 (C-3), 108.9 (C-3a), 119.8 (C-4), 122.4 (C-5), 124.6 (C-6), 112.2 (C-7), 138.0 (C-7a), 32.0 (C-8), 176.5 (C-9)[22] ; FABMS *m*/*z*: 176 $[M+H]^+$.

Uracil (**2**): White solid; mp >250°C; ^{1}HNMR (300 MHz, CD_3OD): δ 7.39 (1H, *d*, 7.5, H-6), 5.61(1H, *d*, 7.5, H-5); ^{13}CNMR (125 MHz, CD_3OD): δ 167.4 (C-4), 153.5 (C-2), 143.6 (C-6), 101.7 (C-5) [4]; FABMS *m*/*z*: 113 $[M+H]^+$.

Results and discussion

A fungal endophyte isolated from unwounded leaves of *P. nigrum* was identified as *Colletotrichum siamense* based on the sequence of internal transcribed spacer (ITS) region of rDNA gene. A pure culture of *C. siamense* was fermented in PDB media. The broth and mycelium were separately extracted with EtOAc. Based on the TLC analysis, two EtOAc extracts were combined and screened for bioactivities. The EtOAc extract displayed antioxiadant activity against DPPH (IC_{50} = 294 ppm), phytotoxicity for lettuce seed germination; shoot and root inhibition (IC_{50} = 195 ppm & 32 ppm respectively) and brine shrimp lethality (IC_{50} = 837 ppm).

Chromatographic separation of the EtOAc extract over silica gel, Sephadex LH-20 and PTLC afforded indole-3-acetic acid (IAA) (**1**) and uracil (2) (Figure 1), which were identified by spectroscopic analysis (^{1}H and ^{13}CNMR) and direct TLC comparison with authentic samples. Compound **1** was tested for antifungal activity against *C. cladosporiodies* using TLC bioautographic method and was found to strongly inhibit the fungal growth.

The minimum quantity of the compound required to inhibit the growth of *C. cladosporioides* was 4 µg/spot while compound **2** was found to be inactive. Further compound **1** showed antioxidant activity in DPPH radical scavenging assay (IC_{50} value of 205 µM). Somewhat higher DPPH radical scavenging activity of IAA (IC_{50} value: 75 µM) was reported previously [7]. IAA is the most common naturally occurring plant hormone of the auxin class, which promotes plant growth, the rooting of cuttings, and the formation of fruit without fertilization. Compound **1** showed negligible activity in brine shrimp toxicity and α-amylase inhibitory assays, thus suggesting the presence of some other compounds having these activities.

This is the first report on the isolation of the endophytic fungus *C. siamense* from unwounded leaves of *P. nigrum*, whereas the fungus has been isolated previously from anthracnose-infected plant of *P. nigrum* [9]. *Colletotrichum* species are well-known plant pathogens and have been isolated from anthracnose of several tropical host plants [9]. Therefore, *C. siamense* can be regarded as a latent pathogen in the leaves of *P. nigrum* as in the case of *C. gloeosporioides* in banana [17]. Fermentation of *C. siamense* in PDB media led to the isolation of plant growth hormone IAA. To our knowledge this is the first report on the isolation of IAA from the rigorously identified *Colletotrichum* species. There are a few reports of IAA production by endophytic fungi, e.g., an unidentified *Colletotrichum* sp. isolated from *Artemisia annua* [13] and *Purpureocillium lilacinum* [5]. IAA displayed antifungal activity against the plant pathogenic fungus *C. cladosporiodies* in accordance with its known antifungal property [1,12].

The present study also confirmed weak antioxidant property of IAA in DPPH radical scavenging assay. IAA is considered to be the major auxin of plants derived from tryptophan controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity. Since IAA has a high global demand in commercially this endophytic fungal source can be used to produce IAA in large scale by metabolic engineering and fermentative production. Uracil is one of the four nucleobases in the nucleic acid of RNA and has been often isolated from fungal species

including endophytic fungi such as *Neofusicoccum* sp [20]. *Colletotrichum gloeosporioides* [6] and *Penicillium commune* [27].

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Conflict of interests

The authors declare that they have no conflict of interest.

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