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Endophytic Fungus *Nigrospora oryzae* from a Medicinal plant *Coccinia grandis*, a High Yielding New Source of Phenazine-1-carboxamide[§]

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[§]*This paper is dedicated to the late Prof. G.P. Wannigama, Sri Lanka, an inspirational scientist and teacher, on the first anniversary of his death.*

Nigrospora oryzae was isolated as an endophytic fungus from the leaves of *Coccinia grandis*, a popular medicinal plant used to control diabetes. Fermentation of the fungus in potato dextrose broth and chromatographic purification of the ethyl acetate extracts of the broth and mycelium yielded two phenazine secondary metabolites, which were identified as phenazine-1-carboxylic acid (**1**) and phenazine-1-carboxamide (**2**) by comparing their spectral data with those reported in the literature. Compound **2**, isolated in high yield (1 g/4 L medium), showed strong antifungal activity against the plant pathogen *Cladosporium cladosporioides*. This is the first report of the isolation of *N. oryzae* as an endophytic fungus of *C. grandis*. These phenazines have never been isolated from any fungal source. Antifungal activity of **2** against *C. cladosporioides* is reported for the first time.

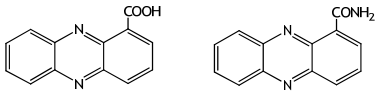
Keywords: *Coccinia grandis*, Endophytic fungi, *Nigrospora oryzae*, Phenazine-1-carboxylic acid, Phenazine-1-carboxamide.

Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically remained unnoticed [1]. Endophytic fungi have been a good source of bioactive compounds [2]. Some endophytic fungi have the ability to produce the same compounds that are produced by their host plant [3]. Camptothecin, huperzine A, podophyllotoxin, taxol, vinblastine and vincristine are some examples of such compounds [4].

In a continuation of our studies directed towards the search for bioactive compounds from Sri Lankan flora, we investigated secondary metabolites produced by an endophytic fungus, *Nigrospora oryzae*, isolated from *Coccinia grandis* of the family Cucurbitaceae. *C. grandis* is a popular medicinal plant used for the treatment of diabetes in Sri Lanka and India [5]. An antifungal agent, griseofulvin [6], and some sesquiterpenes [7] have been reported from *N. oryzae*.

In this paper we report the isolation of an endophytic fungus from the leaves of *C. grandis*, identification of the fungal species as *N. oryzae*, and the isolation of phenazine-1-carboxylic acid (**1**) and phenazine-1-carboxyamide (**2**) as fermentation products. Compound **2** showed antifungal activity against the plant pathogen *Cladosporium cladosporioides*.

The endophytic fungus was isolated on potato dextrose agar (PDA) from the sterilized leaves of *C. grandis* and identified as *N. oryzae* by sequence analysis of the internal transcribed spacer (ITS) region of rDNA. Inoculation of *N. oryzae* culture to potato dextrose broth (PDB), fermentation for 4 weeks and extraction of the broth and mycelium with ethyl acetate (EtOAc) gave the respective EtOAc extracts. The two extracts were combined since they showed similar TLC patterns. The combined extract was active in antifungal assay against *C. cladosporioides* by TLC bioautography [8], brine shrimp toxicity assay against *Artemia salina* (IC_{50} 190 ppm) [9], phytotoxicity assay against *Lactuca sativa* seed germination for inhibiting shoot and root elongation (IC_{50} 653 and 339 ppm, respectively) [10] and antioxidant assay evaluated by DPPH (2,2'-diphenyl-1-picrylhydrazine) radical scavenging ability (EC_{50} 462 ppm) [11].



1 **2**

Figure 1: Structures of Compounds **1** and **2**.

The extract was chromatographed over silica gel, Sephadex LH-20 and preparative TLC (PTLC) to give compounds **1** and **2**, which were identified as phenazine-1-carboxylic acid [12] and phenazine-1-carboxamide [13], respectively by comparison of their spectral data with those reported. Compound **2** was obtained as a major metabolite (2.0 g from 13.6 g of the combined EtOAc extract obtained from 8 L PDB).

Only compound **2** strongly inhibited the growth of *C. cladosporioides*. The minimum quantity of compound **2** required to inhibit the growth of *C. cladosporioides* on a TLC plate was found to be 4 μg/spot. Compound **2** was weakly active in the brine shrimp toxicity assay (IC_{50} 439 μM). Compounds **1** and **2** displayed weak phytotoxicity (IC_{50} 540 and 444 μM for inhibiting root and shoot elongation, respectively). Compounds **1** and **2** did not show significant antioxidant activity.

Phenazines comprise a large group of nitrogen-containing heterocyclic compounds and over 100 biologically active phenazine derivatives have been reported from a variety of bacteria, especially *Pseudomonas* [14]. They have a broad spectrum of antibiotic activity [14]. Compound **1**, also known as tubermycin B [15], is one of the simple molecules of the phenazine family. Phenazine-producing bacteria such as *Pseudomonas* spp. are part of microcolonies (biofilms) and play an important role in the rhizosphere and soil ecosystems. These bacteria compete for

colonization sites on the roots of agricultural crops and protect the plants from pathogenic fungi and bacteria [16].

The endophytic fungus *N. oryzae* was isolated from *C. grandis* for the first time. This is the first report of the isolation of phenazine derivatives from a fungal source. The present study also reported antifungal activity of **2** against the plant pathogenic fungus *C. cladosporioides* for the first time. Furthermore, *N. oryzae* was shown to be a very good producer of phenazine-1-carboxyamide (**2**). It would be interesting to investigate other plant parts further, in particular roots and fruits of *C. grandis*, as a possible source of endophytic fungi, which may produce environmentally friendly fungicides useful for the protection of agricultural crops.

Experimental

Isolation and identification of endophytic fungus: Fresh leaves of *C. grandis* were collected from the Central Province of Sri Lanka in February, 2014. Leaves were rinsed in running water and sterilized with ethanol, 5% NaOCl and distilled water 3 times. A segment (ca. 10 mm x 5 mm) of the leaf was placed on PDA media in a Petri-dish (90 mm) and incubated at room temperature. Emerging fungi were isolated after 4 days and sub-cultured to obtain a pure fungal culture. The fungus was identified as *N. oryzae* by sequence analysis of the ITS region of the rDNA gene. DNA was extracted using Promega, Wizard Genomic DNA Purification Kit (A1120) and amplification of the ITS region was carried out using the universal eukaryotic primers of ITS1 and ITS4. These experiments were performed by the GeneTech Institute, Sri Lanka. BLAST search indicated that the sequence of the ITS region had 100% similarity to that of *Nigrospora oryzae* CEQCA-M1190 (GenBank Accession No. KC771472.1). Photographic evidence of the leaves of *C. grandis* and *N. oryzae* strain (IFS/D/EF1/2014) are deposited at the Institute of Fundamental Studies.

Extraction and isolation of compounds: Large scale culturing of the fungus was carried out by inoculating *N. oryzae* culture grown on PDA medium to 1 L conical flasks (x 20) each containing 400 mL of PDB medium, which were allowed to stand at room temperature for 10 days, and then incubated while shaking (90 rpm) every other day for another 18 days. The medium was filtered and the filtrate was partitioned with EtOAc/H_2O. Concentration of the EtOAc layer gave the extract (6.25 g). The residual mycelium was crushed and extracted with EtOAc to give the EtOAc extract (7.33 g). Both extracts were combined and chromatographed over silica gel (*n*-hexane-CH_2Cl_2-MeOH), Sephadex LH-20 (MeOH) and PTLC (developed with $CHCl_3$-MeOH 20:1, R_f 0.49 for **1** and 0.37 for **2** on silica gel 60 F254 glass plate, Merck) to give compounds **1** (100 mg) and **2** (2.0 g). Purity of **1** and **2** were checked by HPLC [H_2O-MeOH (1:4), UV detection at 254 nm]

Supplementary data: Bioassay procedures and ^1H and ^{13}C NMR spectra of compounds **1** and **2**.

References

[1] Strobel SA, Strobel GA. (**2007**) Plant endophytes as a platform for discovery-based undergraduate science education. *Nature Chemical Biology*, ***3***, 356–359.

[2] Bhardwaj A, Agrawal P. (**2014**) A review fungal endophytes: as a store house of bioactive compounds. *World Journal of Pharmacy and Pharmaceutical Sciences*, ***3***, 228-237.

[3] Strobel GA. (**2003**) Endophytes as sources of bioactive products, *Microbes and Infection*, ***5***, 535–544.

[4] (a) Sachin N, Manjunatha BL, Kumara PM, Ravikanth G, Shweta S, Suryanarayanan TS, Ganeshaiah KN, Shaanker RU. (**2013**) Do endophytic fungi possess pathway genes for plant secondary metabolites? *Scientific Correspondence*, ***104***, 178–182; (b) Zhao J, Zhou L, Wang J, Shan T, Zhong L, Liu X, Gao X. (**2010**) Endophytic fungi producing bioactive compounds originally from their host plants. *Current Research, Technology Education Topics in Applied Microbiology and Microbial Biotechnology*, ***1***, 567–576.

[5] (a) Jayaweera DMA. (**1980**) Medicinal plants used in Ceylon, Part II. The National Science Council of Sri Lanka, Colombo; (b) Mazumder PM, Sasmal D, Nambi RA. (**2008**) Antiulcerogenic and antioxidant effects of *Coccinia grandis* (Linn.) Voigt leaves on aspirin-induced gastric ulcer in rats. *Natural Product Radiance*, ***7***, 15–18.

[6] Rathod DP, Dar MA,Gade AK, Rai MK. (**2014**) Griseofulvin producing endophytic *Nigrospora oryzae* from Indian *Emblica officinalis* Gaertn: a new report. *Austin Journal of Biotechnology and Bioengineering*, ***1***, 5,

[7] Li D, Chen Y, Pan Q, Tao M, Zhang W. (**2014**) A new eudesmane sesquiterpene from *Nigrospora oryzae*, an endophytic fungus of *Aquilaria sinensis*. *Records of Natural Products*, ***8***, 330-333.

[8] Homans AL, Fuchs A. (**1970**) Direct bioautography on thin layer chromatograms as a method for detecting fungitoxic substances. *Journal of Chromatography*, ***51***, 327–329.

[9] Meyer BN, Ferrigini NR, Putnnam JE, Jacobsen LB, Nichols, DE, McLaughlin, JL. (**1982**) Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica*, ***45***, 31–34.

[10] D'abrosca B, Dellagreca M, Fiorentino A, Monaco P, Natale A, Oriano P, Zarrelli A. (**2005**) Structural characterization of phytotoxic terpenoids from *Cestrum parqui*, *Phytochemistry*, ***66***, 2681–2688.

[11] (a) Tepe B, Eminagaoglu O, Akpulat A, Aydin E. (**2007**) Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia verticillata* (L.) subsp. *verticillata* and *S. verticillata* (L.) subsp. *amasiaca* (Freyn & Bornm.). *Food Chemistry*, ***100***, 985–989; (b) Alakolanga AGAW, Kumar NS, Jayasinghe L, Fujimoto Y. (**2015**) Antioxidant property and α-glucosidase, α-amylase and lipase inhibiting activities of *Flacourtia inermis* fruits: Characterization of malic acid as an inhibitor of the enzymes. *Journal of Food Science and Technology*, ***52***, DOI 10.1007/s13197-015-1937-6.

[12] Raio A, Puopolo G, Cimmino A, Danti R, Rocca GD, Evidente A. (**2011**) Biocontrol of cypress canker by the phenazine producer *Pseudomonas chlororaphis* sub sp. *aureofaciens* strain M71. *Biological Control*, ***58***, 133–138.

[13] Kumar RS, Ayyadurai N, Pandiaraja P, Reddy AV, Venkateswarlu Y, Prakash O, Sakthivel, N. (**2005**) Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits, *Journal of Applied Microbiology*, ***98***, 145–154.

[14] Pierson III LS, Pierson EA. (**2010**) Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Applied Microbiology and Biotechnology*, ***86***, 1659–1670.

[15] Rane MR, Sarode PD, Chaudhari BL, Chincholkar SB. (**2001**). Detection, isolation and identification of phenazine-1-carboxylic acid produced by biocontrol strains of *Pseudomonas aeruginosa*. *Journal of Scientific & Industrial Research*, ***66***, 627–631.

[16] Mazzola M, Cook RJ, Thomashow LS, Weller DM, Pierson LS. (**1992**) Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. *Applied and Environmental Microbiology*, ***58***, 2616–2624.