Antimicrobial activity of crude extracts of *Phyllosticta* spp.

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\textit{(Received 23 April 2013; final version received 2 July 2013)}

Four strains of *Phyllosticta* were selected to study their antimicrobial activities. The tested strains were identified as *Phyllosticta capitalensis*, *P. citriasiana*, and *P. cordylinophila* based on the internally transcribed spacer (ITS) sequences and morphologies. Initially, the fungal strains were cultured on the potato dextrose agar (PDA) and cultivated at 27\textdegree C for 3 weeks. After incubation, the fungal metabolites were extracted using ethyl acetate. The crude extracts were then concentrated using evaporation and subsequently used in antimicrobial activity assay. Our results showed that these fungal crude extracts could inhibit the growth of *Escherichia coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa* but were not active against *Aspergillus niger*.

**Keywords:** antimicrobial activity; *Guignardia*; secondary metabolites

### Introduction

*Phyllosticta* species encompass a wide variety of lifestyles being parasitic, endophytic, and saprobic. They can cause leaf spot and fruit rots (Glienke-Blanco et al. 2002; Silva & Pereira 2007). Its teleomorph state *Guignardia* is also widely known as a causal pathogen on Citrus and grapevine (Baayen et al. 2002; Paul et al. 2005). An example of *Phyllosticta* species known to be the causal agent of freckle in Queensland was *Phyllosticta cavendishii* (Wong et al. 2012). The distinct morphological characters of *Phyllosticta* species are the slime layer and apical appendage on conidia (van der Aa et al. 2002; Wikee et al. 2011).

*Phyllosticta* species are commonly known to produce various kinds of secondary metabolites (Wijeratne et al. 2008; Evidente, Cimmino, Andolfi, Vurro, Zonno, Motta, et al. 2008; Evidente, Cimmino, Andolfi, Vurro, Zonno, Cantrell, 2008; Kumaran et al. 2009; Kumaran, Muthumary, Kim, 2009). These metabolites include phyllostin and phyllostoxin. Phyllostictine A exhibiting growth-inhibitory activity in five cancer cell lines has been isolated from *P. cirsi* (Le Calvé et al. 2011). Endophytic fungi in mazonian toxic plants exhibit antimicrobial activity (Sette et al. 2006). Five new metabolites have been reported from *P. spinarum* (Wijeratne et al. 2008), which can potentially be applied in several aspects such as biocontrol, medicine, and pharmaceutical industry. *Phyllosticta* species can therefore be considered as potential sources for discovery of novel active compounds.

As part of the programme to study the diversity of *Phyllosticta* in Thailand, we chose four different strains to determine their antimicrobial property. In addition, the identity of these *Phyllosticta* species was characterized based on morphology and phylogeny inferred from the internally transcribed spacer (ITS) sequences.

### Materials and methods

#### Fungal strains

Four *Phyllosticta* strains, isolated from leaf spot and healthy leaves of ornamental plants (Table 1), were obtained from Mae Fah Luang University Culture Collection (MFLUCC) as follows: *Phyllosticta capitalensis* (strains MFLUCC10-0138 and MFLUCC12-0015) isolated from *Euphobia milli* and *Pyrrosia adnascens*; *Phyllosticta cordylinophila* (strain MFLUCC12-0014) isolated from *Cordyline fruticosa*; and *Phyllosticta citriasiana* (strain MFLUCC10-0137) isolated from *Citrus maxima*.

#### Preparation of fungal extracts

The fungal strains were cultured on the potato dextrose agar (PDA) plates at 27\textdegree C for 3 weeks. For extraction, the culture agar was chopped, diced, and extracted with...
Table 1. List of *Phyllosticta* strains and their hosts used in this study.

| Species             | Host               | Habitat       | Mode of life | Growth rate on PDA at 25°C (cm/day) | Conidia (µm) |
|---------------------|--------------------|---------------|--------------|-------------------------------------|--------------|
| *P. citriasiana*     | *Citrus maxima*    | Fruit peel    | Pathogen     | 0.27                                | 6−12 × 3−8   |
| MFLUCC10-0137       |                    |               |              |                                     |              |
| *P. capitalensis*    | *Pyrossia adnascens*| Healthy leaf  | Endophyte    | 0.36                                | 8−12 × 5−6   |
| MFLUCC10-0138       |                    |               |              |                                     |              |
| *P. cordylinophila*  | *Cordyline fruticosa*| Fresh leaf   | Pathogen     | 0.70                                | 5−12 × 4−6   |
| MFLUCC12-0014       |                    |               |              |                                     |              |
| *P. capitalensis*    | *Euphobia milli*  | Fresh leaf    | Pathogen     | 0.36                                | 8−12 × 5−6   |
| MFLUCC12-0015       |                    |               |              |                                     |              |

Table 2. Antimicrobial activity of *Phyllosticta* species. Data shown are mean ± SD of the clear zone diameter of triplicate experiment.

| Bacterial strains          | *P. citriasiana* MFLUCC 10-0137 | *P. capitilensis* MFLUCC 10-0138 | *P. cordylinophila* MFLUCC 12-0014 | *P. capitilensis* MFLUCC 12-0015 |
|----------------------------|---------------------------------|---------------------------------|-----------------------------------|---------------------------------|
| Gram-positive              |                                 |                                 |                                   |                                 |
| *Bacillus cereus*          | 7.5 ± 0.7                       | 11.5 ± 0.7                      | 7.0 ± 0.0                         | 8.0 ± 1.4                       |
| *Staphylococcus aureus*    | −                               | 7.0 ± 0.0                       | −                                 | 7.0 ± 0.0                       |
| *Micrococcus luteus*       | −                               | 10.5 ± 0.7                      | −                                 | 8.0 ± 1.4                       |
| Gram-negative              |                                 |                                 |                                   |                                 |
| *Escherichia coli*         | −                               | 9.5 ± 0.7                       | −                                 | 8.5 ± 0.7                       |
| *Pseudomonas aeruginosa*   | −                               | 8.0 ± 0.0                       | 7.0 ± 0.0                         | 7.0 ± 0.0                       |
| *Salmonella typhimurium*   | −                               | 9.0 ± 1.4                       | −                                 | 7.0 ± 0.0                       |

Note: (−) = no inhibition activity.

Figure 1. Effect of *Phyllosticta* crude extracts on *Bacillus cereus* (left) and *E. coli* (right).

Note: *Phyllosticta* strains were indicated as culture collection numbers: 10–0137, 10–0138, 12–0014, and 12–0015 (See Tables 1 and 2 for further details).

10 ml ethyl acetate (per plate), and homogenized using a blender (high speed for 3 min). The organic solution was then covered with foil and incubated at room temperature for 24 h. The organic solution was collected through filtration and the remaining agar residue was extracted successively using the same solvent until the filtrate was colorless. The filtrates were then combined and concentrated by evaporating at 60° C, yielding a crude yellow syrupy extract. These crude extracts (~2 ml) were kept at 4°C until use.

**Antimicrobial activity assay**

The fungal crude extracts were tested for antimicrobial activity against the following microbes: *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia...
coli, Pseudomonas aeruginosa, Salmonella typhimurium, and a fungus Aspergillus niger. The antimicrobial activity was then performed using a disc diffusion assay (Kirby et al. 1957). A sterilized disc paper with a diameter of 0.6 cm was soaked in the fungal crude extracts for 2 h and transferred to the lawn of tested microorganisms. The diameter of the clear zones was then recorded.

Identification of fungal strains

Initially, the morphology of the fungal strains was determined as previously described (Wikee et al. 2013). Besides, the fungal identity was further confirmed using the ITS sequence analysis. For this, the fungal genomic DNA was extracted and subsequently used as a template in the polymerase chain reaction (PCR). To amplify the ITS region, the primers used were V9G and ITS4 (White et al. 1990; De Hoog & Gerrits van den Ende 1998). The PCR reaction system consists of DNA template (∼500 ng), 1.5 mM MgCl₂, 1X Taq buffer, 200 µM dNTPs, 0.01 µl of V9G and ITS4 primers, and 0.05 µl of Taq DNA polymerase. The amplified products were then sequenced by an automatic DNA sequence (Perkin-Elmer, Norwalk, CT, United States) (CBS Fungal Biodiversity Centre Uppsalalaan 8, 3584 Utrecht, Netherlands). The obtained nucleotide sequence data were deposited in the GenBank database with the following accession numbers: KC686597 for *P. capitulensis* MFLUCC 10–0138, KC686598 for *P. capitulensis* MFLUCC 12–0015, KC686600 for *P. citriasiana* MFLUCC 10–0137, and KC686599 for *P. cordylinophila*. These DNA sequences were then analyzed using MEGA v5.05 software (Tempe, AZ, USA; Tamura et al. 2011). The phylogenetic tree was constructed using PAUP version 4.0b10 (Sunderland, MA, USA; Swofford 2002).

| Species                  | Strain code number | GenBank accession number |
|--------------------------|--------------------|--------------------------|
| *G. mangiferae*          | IMI 260576         | JF261459                 |
| *P. braziliiana*         | LGMF 333           | JF343374                 |
| *P. braziliiana*         | LGMF 334           | JF343366                 |
| *P. braziliiana*         | LGMF 330           | JF343572                 |
| *P. capitulensis*        | CPC 20251          | KC291333                 |
| *P. capitulensis*        | CPC 20252          | KC291334                 |
| *P. capitulensis*        | MFLUCC12-0015      | KC6866598                |
| *P. capitulensis*        | MFLUCC10-0138      | KC686597                 |
| *P. capitulensis*        | CBS 100175         | FJ383320                 |
| *P. capitulensis*        | CBS 114751         | EU167584                 |
| *P. capitulensis*        | CBS 115046         | FJ383322                 |
| *P. capitulensis*        | CBS 115047         | FJ383323                 |
| *P. capitulensis*        | CBS 115049         | FJ383324                 |
| *P. capitulensis*        | CBS 123373         | FJ383331                 |
| *P. capitulensis*        | CBS 123404         | FJ383333                 |
| *P. capitulensis*        | LGMF 03            | JF261452                 |
| *P. capitulensis*        | LGMF 181           | JF261447                 |
| *P. capitulensis*        | LGMF 219           | JF261448                 |
| *P. capitulensis*        | LGMF 240           | JF261443                 |
| *P. capitulensis*        | LGMF 222           | JF261450                 |
| *P. capitulensis*        | LGMF 220           | JF261446                 |
| *P. capitulensis*        | LGMF 358           | JF261449                 |
| *P. capitulensis*        | CPC18848           | JF261465                 |
| *P. citriasiana*         | CBS 120486         | FJ383360                 |
| *P. citriasiana*         | CBS 123370         | FJ383355                 |
| *P. citriasiana*         | CBS 123371         | FJ383356                 |
| *P. citriasiana*         | CBS 123372         | FJ383357                 |
| *P. citribraziliensis*   | CBS 100098         | FJ383352                 |
| *P. citribraziliensis*   | LGMF09             | JF261436                 |
| *P. citricarpa*          | CBS 102374         | FJ383313                 |
| *P. citricarpa*          | CBS 120489         | FJ383315                 |
| *P. citricarpa*          | CBS 127454         | JF343583                 |
| *P. citricarpa*          | CBS 127452         | JF343581                 |
| *P. citricarpa*          | CBS 127455         | JF343584                 |
| *P. citrichinaensis*     | ZJUCC 200956       | JN791664                 |
| *P. citrichinaensis*     | ZJUCC 200964       | JN791662                 |
| *P. citrichinaensis*     | ZJUCC 2010150      | JN791620                 |
| *P. citrichinaensis*     | ZJUCC 2010152      | JN791611                 |
| *P. cordylinophila*      | MFLUCC12-0014      | KC686599                 |
| *P. cordylinophila*      | MUC 521            | AB543537                 |
| *P. cussonia*            | CPC 14873          | JF343578                 |
| *P. hypoglossi*          | CBS 101.72         | FJ383365                 |
| *P. hypoglossi*          | CBS 434.92         | FJ383367                 |
| *P. hypoglossi*          | CBS 167.85         | FJ383366                 |
| *P. spinarum*            | CBS 292.90         | JF343585                 |
| *B. obtusa*              | CMW 8232           | AY972105                 |

Notes: CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: working collection of Pedro Crous housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Berkshire; LGMF: culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil; ZJUCC: Zhejiang University Culture Collection, Zhejiang, China.
Results and discussion

Although *Phyllosticta* species are widely known to produce useful metabolites, the study of their antimicrobial activity was limited to certain species (Evidente, Cimmino, Andolfi, Vurro, Zonno, Motta, et al. 2008; Kumaran, Muthumary, Kim, et al. 2009). These strains were selected...
based on their distinct characteristics (i.e., new species, host specificity, and mode of life). This preliminary study is expected to provide results that can be used as a guideline, and thus further studies on antibacterial activities of *Phyllosticta* will be explored.

Table 2 shows the antimicrobial activity of crude extracts obtained from four different *Phyllosticta* strains. These fungal extracts showed inhibitory effects against at least one of the testing microbes with diameters of the clear zones ranging from 7 to 11.5 mm. Interestingly, the extracts derived from *P. capitulensis* exhibited not only the highest antibacterial activity but also a wide range of inhibitory activity against both Gram-positive and Gram-negative bacteria used in the test (Figure 1). It should also be noted that the extracts produced from different strains of *P. capitulensis* (MFLUCC 10–0138 and MFLUCC 12–0015) resulted in different profiles in antibacterial activity results. However, none of these fungal extracts could inhibit the growth of the fungus *Aspergillus niger*. These fungal extracts will be subjected to further analysis as it has been reported that several *Phyllosticta* species can produce a wide range of secondary metabolites with diverse biological activities (Peláez et al. 1998; Radu & Kqueen, 2002; Ezra et al. 2004; Strobel et al. 2004; Gangadevi & Muthumary, 2008; Wijeratne et al. 2008; Srinivasan et al. 2010). The fungal strains were characterized based on phenotypic characters. This included the growth rates, cultural features, and cell morphologies. In general, colors of these *Phyllosticta* colonies were black when cultured on PDA, with white or dark green for their mycelium depending on the age of the fungal cultures (Figure 2). When using malt extract agar, the fungal strains initially grew with white mycelium subsequently turned to greenish or dark green (or black). Table 2 summarizes some morphological characters as well as a source of isolation of the four *Phyllosticta* strains used in this study. It should be noted, however, that the sole use of morphology is inadequate for identifying *Phyllosticta* to species level (Su & Cai 2012). Molecular approach was then introduced to further confirm the species identity. In this study, the ITS sequences of these fungal strains were used in a phylogenetic analysis incorporating the known sequences of 43 *Phyllosticta* strains from GenBank (Table 3). Based on this analysis, the four *Phyllosticta* strains were identified as follows: MFLUCC 10–0137 as *P. citriasiana*, MFLUCC 12–0014 as *P. cordylinophila*, MFLUCC 10–0138, and MFLUCC 12–0015 as *P. capitulensis* (see Figure 3).

In conclusion, the crude extracts of these *Phyllosticta* strains exhibited a broad spectrum of activity against Gram-positive and Gram-negative bacteria responsible for the most common bacterial diseases and normal flora on the human skin. *Phyllosticta* species are obviously a rich and reliable source of bioactive and chemically novel compounds with significant medicinal and agricultural potential.

**Acknowledgments**

This study was financially supported by the Asia Research Centre, Chulalongkorn University (No. 001/2556), and the National Research Council of Thailand (No. 55201020002).

**References**

Baaren R, Bonants P, Verkley G, Carroll G, Van Der Aa H, De Weerdt M, Van Brouwershaven SG, Maccheroni, W, Jr, De Blanco C. 2002. Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitulensis*). Phytopathology. 92: 464–477.

De Hoog GS, Gerrits Van Den Ende AHG. 1998. Molecular diagnostics of clinical strains of filamentous Basidiomycetes. Mycoses. 41:183–189.

Evidente A, Cimmino A, Andolfi A, Varro M, Zonno M, Cantrell C, Motta A. 2008b. Phyllostictine A–D, oxazatricycloalkenones produced by *Phyllosticta cirsii*, a potential mycoherbicide for *Cirsium arvense* biocontrol. Tetrahedron. 64:1612–1619.

Evidente A, Cimmino A, Andolfi A, Varro M, Zonno M, Motta A. 2008. Phyllostoxin and phyllostin, bioactive metabolites produced by *Phyllosticta cirsii*, a potential mycoherbicide for *Cirsium arvense* biocontrol. J Agric Food Chem. 56:884–888.

Ezra D, Hess WM, Strobel AG. 2004. New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus. Microbiology. 150:4023–4031.

Gangadevi V, Muthumary J. 2008. A simple and rapid method for the determination of taxol produced by fungal endophytes from medicinal plants using high performance thin layer chromatography. Chin J Chrom. 26:50–55.

Glenike-Blanco C, Aguilar-Vildoso CI, Vieira MLC, Barroso PA, Azevedo JL. 2002. Genetic variability in the endophytic fungus *Guignardia citricarpa* isolated from citrus plants. Genet Mol Biol. 25:251–255.

Kirby WMM, Yoshihara GM, Sundsted KS, Warren JH. 1957. Clinical usefulness of a single disc method for antibiotic sensitivity testing. Antibiot Annu. 1956–1957:892.

Kumaran RS, Muthumary J, Hur BK. 2009. Isolation and identification of an anticancer drug, taxol from *Phyllosticta tabernemontanae*, a leaf spot fungus of an angiosperm, *Wrightia tinctoria*. Microbiology. 47:40–49.

Kumaran RS, Muthumary J, Kim EK, Hur BK. 2009. Production of taxol from *Phyllosticta dioscoreae*, a leaf spot fungus isolated from *Hibiscus rosa-sinensis*. Biotechnol Bioprocess Eng. 14:76–83.

Le Calvé, B, Lallemant B, Perrone C, Lenglet G, Depauw S, Van Goetsenov G, Bury M, Vurro M, Herphelin F, Andolfi A. 2011. *In vitro* anticancer activity, toxicity and structure – activity relationships of phyllostictine A, a natural oxazatricycloalkenone produced by the fungus *Phyllosticta cirsii*. Toxicol Appl Pharm. 25:8–17.

Paul I, Van Jaarsveld AS, Korsten L, Hattingh V. 2005. The potential global geographical distribution of citrus black spot caused by *Guignardia citricarpa* (Kieyl): likelihood of disease establishment in the European Union. Crop Prot. 24:297–308.

Peláez F, Collado JF, Arenal A, Basilio A, Cabello MT, Diaz Matas JB, Garcia A, Gonzalez DV, Gonzalez V, Gorrochategui J, et al. 1998. Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycol Res. 102:755–761.
Radu S, Kqueen CY. 2002. Preliminary screening of endophytic fungi from medicinal plants in Malaysia for antimicrobial and antitumor activity. Malays J Med Sci. 9:23–33.
Sette LD, Passarini MRZ, Delarmelina C, Salati F, Duarte MCT. 2006. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. World J Microbiol Biotechnol. 22:1185–1195.
Silva M, Pereira OL. 2007. First report of Guignardia endophyllicola leaf blight on Cymbidium (Orchidaceae) in Brazil. Aust Plant Dis Notes. 2:31–32.
Srinivasan K, Jagadish LK, Shenbhagaraman R, Muthumary J. 2010. Antioxidant activity of endophytic fungus Phyllosticta sp. isolated from Guazuma tomentosa. J Phytol. 2:37–41.
Strobel GA, Daisy BH, Castillo U, Harper J. 2004. Natural products from endophytic microorganisms. J Nat Prod. 67:257–268.
Su YY, Cai L. 2012. Polyphasic characterisation of three new Phyllosticta spp. Persoonia. 28:76–84.
Swofford D. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4. Sunderland (MA): Sinauer Associates.
Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 28:2731–2739.
Van Der Aa VHS, Aptroot A, Summerbell R, Verkley G. 2002. A revision of the species described in Phyllosticta. Utrecht: Centraalbureau voor Schimmelcultures.
White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego (CA): Academic Press; p. 315–322.
Wijeratne EMK, Paranagama PA, Marron MT, Gunatilaka MK, Arnold AE, Gunatilaka AAL. 2008. Sesquiterpene quinones and related metabolites from Phyllosticta spinarum, a fungal strain endophytic in Platycladus orientalis of the Sonoran Desert (1). J Nat Prod. 71:218–222.
Wikee S, Lombard L, Crous PW, Nakashima C, Motohashi K, Chukeatirote E, McKenzie HCE, Wijnand SJ, Hyde KD. 2013. Phyllosticta capitensis, a widespread endophyte of plants. Fungal Divers. 60:91–105.
Wong MH, Crous PW, Henderson J, Groenewald JZ, Drenth A. 2012. Phyllosticta species associated with freckle disease of banana. Fungal Divers. 56:173–187.