African Journal of Biotechnology Vol. 10(79), pp. 18174-18178, 12 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1801
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Botrallin from the endophytic fungus *Hyalodendriella* sp. Ponipodef12 and its antimicrobial activity

Lingyun Zhong[1], Liang Xu[1], Xiangjie Meng[1], Youliang Peng[1], Yuanquan Chen[1], Peng Sui[1], Mingan Wang[2] and Ligang Zhou[1]*

[1]College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China.
[2]College of Science, China Agricultural University, Beijing 100193, China.

Accepted 4 November, 2011

Bioassay-guided fractionation of the crude methanol extract of the mycelia from the endophytic fungus *Hyalodendriella* sp. Ponipodef12, associated with the hybrid 'Neva' of *Populus deltoides* Marsh × *P. nigra* L., led to the isolation of one compound coded as P12-1 which was identified as botrallin (1,7-dihydroxy-3,9- dimethoxy-4a-methyl-6*H*- dibenzo [*b,d*] pyran-2,6 (4a*H*) -dione) by spectroscopic and physicochemical means. The median inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) of botrallin against *Bacillus subtilis* were 98.47 µg/ml and 200 µg/ml, respectively. The IC_{50} values of botrallin against spore germination of *Magnaporthe oryzae* and *Botryosphaeria dothidea* were 81.62 µg/ml and 110.02 µg/m, respectively. This is the first report about botrallin and its antimicrobial activity from the endophytic fungus *Hyalodendriella* sp. Ponipodef12 derived from *Populus* species.

Key words: Poplar hybrid 'Neva', endophytic fungus, *Hyalodendriella* sp., botrallin, antimicrobial activity.

INTRODUCTION

Plant endophytic fungi are defined as the fungi that live asymptomatically within plant tissues (Zhang et al., 2006; Rodriguez et al., 2009). They have been found in each plant species. During the long period of co-evolution, a friendly relationship was formed between each endophyte and its host plant (Zhao et al., 2011). Host plant can supply plenteous nutriment and easeful habitation for the survival of its endophytes. On the other hand, the endophytes would produce various bioactive constituents for helping their host plants to resist external biotic and abiotic stresses, and benefiting for the host growth in return (Silvia et al., 2007). Otherwise, the secondary metabolites produced by plant endophytic fungi usually display antimicrobial, insecticidal, cytotoxic, and anti-cancer activities, which indicate the tremendous potential of endophytic fungi applying in agriculture, medicine and food industry (Strobel, 2003; Aly et al., 2010; Zhao et al., 2010; Zhou et al., 2010).

Species and hybrids of *Populus* (Salicaceae) are of worldwide importance in the production of fibre and energy (Nixon et al., 2001). Many bioactive compounds such as phenolic acids, flavonoids, and terpenoids have been isolated from *Populus* species (Radoykova et al., 2010; Schnitzler et al., 2010; Dudonne et al., 2011; Zhong et al., 2011). The poplar cultivar 'Neva', hybrid of *Populus deltoides* Marsh × *P. nigra* L., is one of the most important salicaceous woody plants in subtropical and temperate regions with its desirable traits such as drought, insect and disease resistances (Fang et al., 2007; Zhou et al., 2008). To the best of our knowledge, there is no reported study on the endophytic fungi and their secondary metabolites associated with the poplar cultivar 'Neva' though there were some reports about the endophytic fungi from other species or hybrids of *Populus* species (Hutchison, 1999; Xin et al., 2009; Albrectsen et al., 2010).

In this work, we report an endophytic botrallin-producing fungus *Hyalodendriella* sp. Ponipodef12 associated with the poplar hybrid 'Neva'. The antimicrobial activity of

*Corresponding author. E-mail: lgzhou@cau.edu.cn.

botrallin was also evaluated.

MATERIALS AND METHODS

General

Melting point of the compound was determined on an XT4-100B microscopic melting-point apparatus (Tianjin Tianguang Optical Instruments Company, China) and uncorrected. NMR spectra were recorded on a Bruker Avance DRX-400 (^1H at 400 MHz and ^{13}C at 100 MHz) NMR spectrometer using tetramethylsilane (TMS) as an internal standard. HR-ESI-MS spectrum was recorded on a Bruker Apex IV FTMS mass spectrometer. The microplate spectrophotometer (PowerWave HT, BioTek Instruments, USA) was employed to measure light absorption values of the samples. Silica gel (200-300 mesh) for column chromatography (CC) and silica gel GF_{254} for TLC were from Qingdao Marine Chemical Ltd., China. Spots of TLC were visualized by spraying 5% sulphuric acid (H_2SO_4) in EtOH (v/v) followed by heating at 110ºC for 10 min. Both Sephadex LH-20 and silica gel RP-18 for CC were purchased from Pharmacia Biotech, Sweden. All chemicals used in the study were of analytical grade.

Plant materials

The healthy twigs and barks of 5-year-old hybrid poplar 'Neva' of *P. deltoides* Marsh × *P. nigra* L. (Salicaceae) were collected from Longhua Town in Hebei Province of China in June 2009, and were authenticated by Prof. Yuying Xiang of the Institute of Forestry, Chinese Academy of Forestry, where the voucher specimen of this plant was deposited. The plant samples were stored in sealed plastic bags at 4ºC until required.

Isolation and culture of the fungal endophytes

The twigs and barks were washed in running water first, then surface sterilized by soaking in 75% ethanol for 2 min, followed by immersing in 0.2% mercuric chloride for 20 min, and finally rinsed in sterile distilled water for three times (that is 1 min for each time). After surface sterilization, the twigs and barks were eliminated epidermis and cut into the segments (5 × 5 mm), which were placed in 90 mm diameter Petri-dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin sulfate (500 mg/L) to suppress bacteria growth. Plant segments were incubated in the dark at 25ºC for about 1 month, and examined periodically. When the fungal colonies developed, they were transferred to new Petri-dishes with PDA, and subcultured to get pure cultures at last.

Morphological characterization

The morphological characters of the fungal isolate Ponipodef12 were observed and described according to the method of Photita et al. (2005). The growth rate of the fungal colonies on PDA plates was recorded after one week along two present diametrical lines. Morphological identification was referenced by Ainsworth et al. (1973).

DNA extraction, ITS-rDNA amplification and sequence analysis

Total genomic DNA of the fungal isolate Ponipode12 was prepared according to a modification of the rapid preparation of DNA from filamentous fungi (Raeder and Broda, 1985). Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3'), as well as ITS-rDNA amplification were referenced by our previous reports (Xu et al., 2008; Li et al., 2008). For identification, the PCR products were purified using the QIA quick Gel Extraction Kits (Qiagen, Hilden, Germany) and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer. Then the sequence was run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and it was submitted to GenBank where the accession number was obtained.

Mycelial suspension culture and crude extract preparation

A 1000 ml Erlenmeyer flask containing 300 ml of potato dextrose broth (PDB) medium was inoculated with 2 to 3 agar plugs containing mycelia taken from the Ponipode12 cultures in PDA. All flasks were incubated at 150 rpm on a rotary shaker at 25ºC for 15 days. A total of 24 L fermentation broth was obtained. The culture broth was filtrated in vacuum to afford the filtrate and mycelia. The filtrate was concentrated to a small volume, and then fractionated with ethyl acetate to afford filtrate ethyl acetate fraction (3.6 g). The mycelia were lyophilized and powdered (120.2 g), and then extracted with ultrasound in methanol for five times (2 h for each time). The methanol solution was combined and concentrated in vacuum at 50ºC to obtain mycelia methanol crude extract (58.6 g), which was further dissolved in water, and fractionated with petroleum ether and ethyl acetate in turn to obtain petroleum ether fraction (27.6 g) and ethyl acetate fraction (15.8 g), respectively.

Separation and identification of botrallin

By TLC-bioautography assay, ethyl acetate fraction (15.8 g) from the crude methanol extract of the mycelia exhibited stronger antimicrobial activity than any other solvent fractions (that is mycelia petroleum ether fraction, and filtrate ethyl acetate fraction), it was further subjected to chromatography over a silica gel column eluting with a gradient of petroleum ether-acetone (from 100:0 to 0:100, v/v) to afford 16 subfractions. Of them, subfraction 5 (40 mg) was re-subjected to silica gel column chromatography, reversed phase chromatography (RP-18), and further purified over Sephadex LH-20 and recrystallization to afford a pure compound P12-1 (13 mg).

Antimicrobial activity assay

Thin layer chromatography (TLC)-bioautography assay of the samples was carried out according to the method of Zhao et al. (2008). Antimicrobial activity of botrallin was evaluated by both antibacterial and antifungal activity assays. A modified broth dilution-colorimetric assay by using the chromogenic reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA) was used to detect antibacterial activity of the samples according to our previous report (Liu et al., 2010). *Bacillus subtilis* ATCC 11562 was selected for antibacterial activity assay. The spore germination assay by using both rice blast fungus *Magnaporthe oryzae* (strain 131) and poplar stem blister canker pathogen *Botryosphaeria dothidea* was employed to detect the antifungal activity of the samples (Liu et al., 2009; Zhou et al., 2008). The minimum inhibitory concentration (MIC) value to *B. subtilis* was defined as the lowest sample concentration that inhibited visible growth, as indicated by the MTT staining. The median inhibitory concentration (IC_{50}) value was calculated using the linear relation between the inhibitory probability and concentration logarithm according to the method of Sakuma (1998).

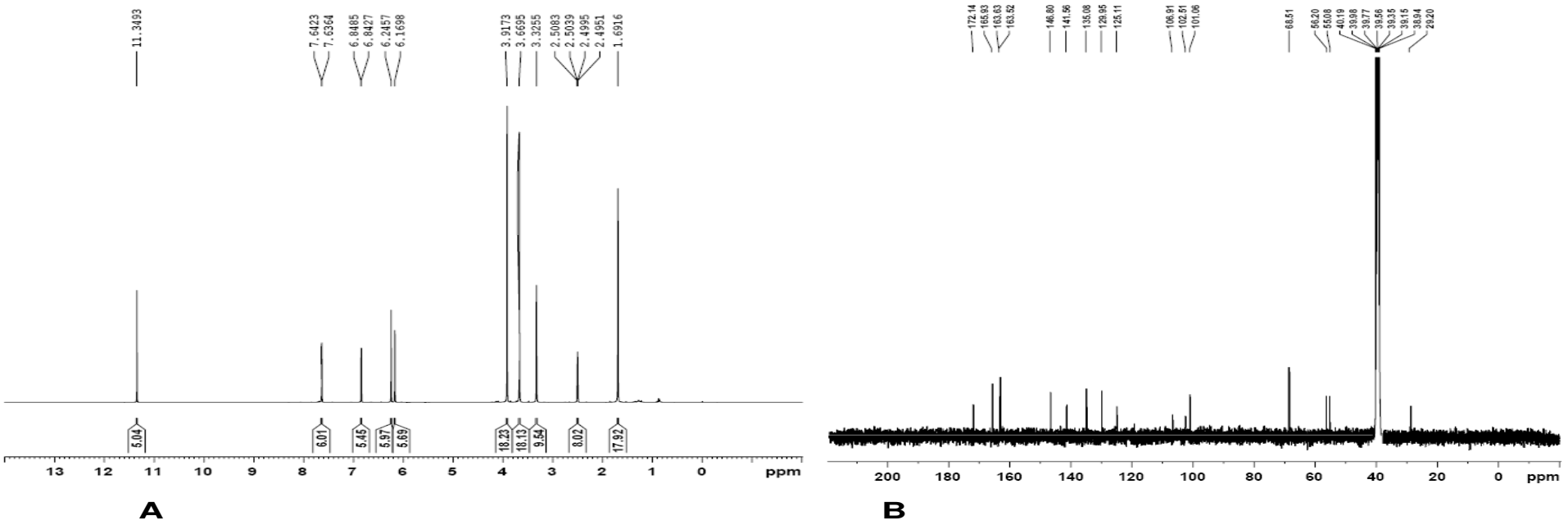

Figure 1. ^{1}H-NMR spectrum (A) at 400 MHz and ^{13}C-NMR spectrum (B) at 100 MHz of botrallin in DMSO-d_6. The chemical shifts were in ppm with TMS as an internal standard.

RESULTS AND DISCUSSION

Screening and identification of the endophytic fungus Ponipodef12

Forty-three endophytic fungal isolates from the twigs and barks of the hybrid poplar 'Neva' of *Populus deltoides* Marsh × *P. nigra* L. were screened for antimicrobial activity by TLC-bioautography assay. Among these fungi, 15 isolates exhibited significant antimicrobial activity against bacteria and fungi (data not shown), and isolate Ponipodef12 showed the greatest antimicrobial activity.

The mycelium of Ponipodef12 consisted of septate, highly branched, 1.5-2.0 µm wide hyphae, smooth, hya-line to pale brown. The colony on PDA spread somewhat erumpent in the centre, with even, catenulate margins, lacking aerial mycelium; surface fuscous-black to olivaceous-black, with patches of cream; grew up to 3.0 cm diameter after one week at 25°C in the dark. Conidiophores formed as the lateral branches on hyphae, subhyaline to pale brown, smooth, 1-6 septate. Conidio-genous cells were terminal. Conidia were 1-2-septate, subcylindrical, straight to curved, subhyaline to pale brown, smooth. The ITS1-5.8S-ITS4 (ITS) partial sequence of Ponipodef12 was submitted to NCBI GenBank to obtain its accession number as HQ731647. The closest sequence similarity of Ponipodef12 was 97% to the fungus *Hyalodendriella* sp. (accession number EU040232 in GenBank). On the basis of the ITS sequence and morphological traits, the fungus Ponipodef12 was considered as the member of the genus *Hyalodendriella*, and identified as *Hyalosdendriella* sp. Ponipodef12 (Crous et al., 2007).

Structural identification of botrallin

One compound (P12-1) was separated from the ethyl acetate fraction of the crude methanol extract of the mycelia based on bioassay-guided fractionation. Its physicochemical and spectrometric data were given as follows: yellowish crystals (MeOH); m.p. 223-227°C; The formula was assigned as $C_{16}H_{14}O_7$ by HR-ESI-MS (*m/z* 319.0814 $[M+H]^+$; calc. 319.0813); ^{1}H-NMR (DMSO-d_6, 400 MHz, Figure 1A) δ (ppm), 6.25

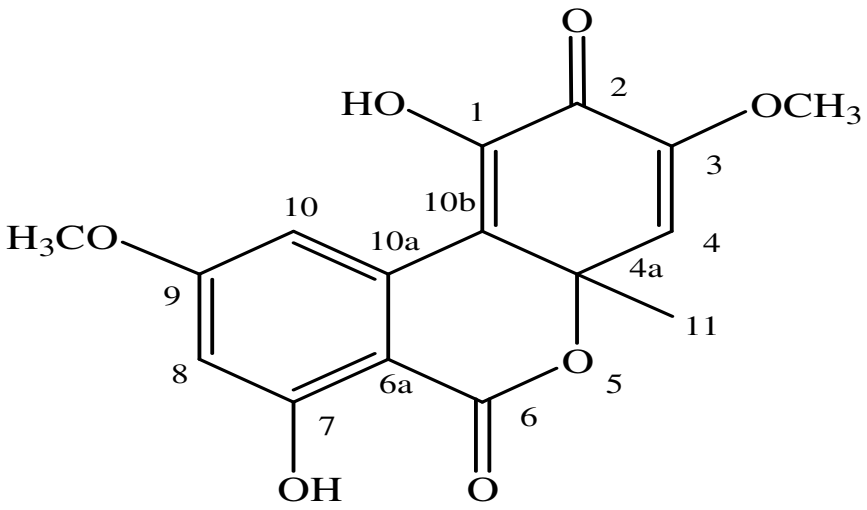

Figure 2. Chemical structure of botrallin (1,7-dihydroxy-3,9-dimethoxy- 4a-methyl-6*H*-dibenzo [*b,d*] pyran-2,6 (4a*H*)- dione).

Table 1. Antimicrobial activity of botrallin.

Test microorganism	Botrallin (μg/ml)		CK^+ (μg/ml)	
	MIC	IC_{50}	MIC	IC_{50}
Bacillus subtilis	200	98.47	50	18.58
Magnaporthe oryzae	nd	81.62	nd	2.00
Botryosphaeria dothidea	nd	110.02	nd	1.89

Streptomycin sulfate was used as the positive control (CK^+) on *Bacillus subtilis*, and carbendazim as CK^+ on *Magnaporthe oryzae* and *Botryosphaeria dothidea*. The 'nd' means not detected.

(1H, s, HO-1), 3.67 (3H, s, CH_3O-3), 6.17 (1H, s, H-4), 11.35 (1H, s, HO-7), 6.84 (1H, d, *J* = 2.36 Hz, H-8), 3.92 (3H, s, CH_3O-9), 7.64 (1H, d, *J* = 2.36 Hz, H-10), 1.69 (3H, s, H-11); ^{13}C-NMR (DMSO-d_6, 100 MHz, Figure 1B) δ (ppm), 141.6 (C-1), 172.1 (C-2), 146.8 (C-3), 55.1 (CH_3O-3), 125.1 (C-4), 68.5 (C-4a), 163.6 (C-6), 101.1 (C-6a), 163.5 (C-7), 102.5 (C-8), 165.9 (C-9), 56.2 (CH_3O-9), 106.9 (C-10), 135.1 (C-10a), 130.0 (C-10b), 29.2 (C-11). After comparing the data with those reported in the literature (Kameda et al., 1974; Hormazabal et al., 2005), P12-1 was identified as botrallin (1,7-dihydroxy-3,9-dimethoxy-4a-methyl-6*H*- dibenzo [*b,d*] pyran-2,6 (4a*H*)- dione) which structure is shown in Figure 2.

Antimicrobial activity of botrallin from the fungus Ponipodef12

Botrallin isolated from the mycelia of Ponipodef12 was assayed for its antimicrobial activity (Table 1). The median inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) values of botrallin against *Bacillus subtilis* were 98.47 and 200 μg/ml, respectively. The IC_{50} values of botrallin against spore germination of *Magnaporthe oryzae* and *Botryosphaeria dothidea* were 81.62 and 110.02 μg/ml, respectively. Botrallin showed moderate antimicrobial activity comparing with the positive control.

Conclusion

Botrallin as the antimicrobial compound was separated and structurally identified from the endophytic fungus *Hyalodendriella* sp. Ponipodef12 associated with the hybrid 'Neva' of *Populus deltoides* Marsh × *P. nigra* L. in this study. Botrallin was firstly isolated from the fungus *Botrytis allii* by Kameda et al. (1974), and later it was isolated from the endophytic fungus *Microsphaeropsis olivacea* associated with a Chilean cupressaceous plant *Pilgerodendron uviferum* by Hormazabal et al. (2005). The MIC value of botrallin on the fungus *Alternaria alternata* was 62.5 μg/ml, and its IC_{50} on the enzyme acetylcholinesterase (AChE) was 6.1 μg/ml. The present study reveals that the endophytic fungus *Hyalodendriella* sp. Ponipodef12 from poplar tree could produce botrallin which showed antimicrobial activity especially on poplar pathogenic fungus *Botryosphaeria dothidea*. It suggests

that the endophytic fungus *Hyalodendriella* sp. Ponipodef12 could have its physiological and ecological roles in helping host plant to resist pathogenic microbes and insects, which need to be further confirmed. From TLC-bioautography examination, some other antimicrobial compounds have not been isolated from the endophytic fungus *Hyalodendriella* sp. Ponipodef12. Separation and purification of these compounds are in progress. In addition, species identification of Ponipodef12 in genus *Hyalodendriella* needs further investigation.

ACKNOWLEDGMENTS

This work was co-financed by the grants from the Program for Changjiang Scholars and Innovative Research Team in University of China (IRT1042) and the National Natural Science Foundation of China (30871662 and 31071710).

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