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Full Length Research Paper

Endophytic fungi from the hybrid 'Neva' of *Populus deltoides* Marsh × *Populus nigra* L. and their antimicrobial activity

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Forty-three endophytic fungal isolates were separated from the healthy twigs and barks of the hybrid poplar 'Neva' of *Populus deltoides* Marsh × *Populus nigra* L. Eleven distinct isolates were selected for further taxonomical identification by morphological traits and internal transcribed spacer (ITS) rRNA gene sequence analysis. Eight genera namely *Alternaria*, *Aureobasidium*, *Botryosphaeria*, *Hyalodendriella*, *Mycosphaerella*, *Peyronellaea*, *Phoma* and *Valsa* were identified on the basis of their morphological characterizations. Of them, the most frequent genus was *Alternaria* (that is, Ponipodef01, Ponipodef04 and Ponipodef07). Their ITS-rDNA sequences were compared with those available in the GenBank database to obtain the closest related species by BLAST analysis. A modified thin layer chromatography-bioautography assay was used to detect the antimicrobial activity of the *n*-butanol extracts of mycelia and culture filtrates of the isolates. Most of the fungal isolates were observed to have antibacterial activity, and antibacterial components mainly existed in mycelia. Two isolates Ponipodef05 and Ponipodef12 were also observed to have antifungal activity. The endophytic fungi isolated from the hybrid poplar 'Neva' of *P. deltoides* Marsh × *P. nigra* L. may be used as potential producers of antimicrobial natural products.

Key words: Poplar hybrid 'Neva', endophytic fungi, extract, antimicrobial activity.

INTRODUCTION

Plant endophytic fungi are defined as the fungi which spend the whole or part of their lifecycle colonizing inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Wilson, 1995; Zhang et al., 2006; Rodriguez et al., 2009). Some endophytic fungi have been detected to produce bioactive metabolites that would be involved in relations between the endophytes and their host plants (Zhao et al., 2011). Many metabolites produced by the endophytes have been tested to have their potential applications as medicinal and agrochemical candidates (Strobel, 2003; Huang et al., 2009; 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). The poplar cultivar 'Neva', which is the hybrid of *Populus deltoides* Marsh × *Populus nigra* L., is one of the most important salicaceous woody plants in subtropical and temperate regions with its desirable characteristics 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 associated with the poplar hybrid

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'Neva'. The objective of this study was to isolate and identify the endophytic fungi from the poplar hybrid 'Neva' as well as to examine their antimicrobial activities.

MATERIALS AND METHODS

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 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 4°C 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 a 90 mm diameter Petri-dish containing potato dextrose agar (PDA) medium supplemented with streptomycin sulfate (500 mg/L) to suppress bacteria growth. After incubation in the dark at 25°C for about 1 month, the segments were examined periodically. When fungal colonies developed, they were transferred to new Petri-dishes with PDA, the fungi were isolated and subcultured to get a pure culture at last. The colonization frequency (CF) of each endophyte was calculated according to the method of Hata and Futai (1995), where CF = (N_{COL}/N_t) × 100 where N_{COL} is the number of segments colonized by each fungus and N_t is the total number of segments.

Morphological characterization

The morphological characters of the fungal isolates were observed and described according to the method of Photita et al. (2005). Morphological identification according to standard taxonomic key included colony diameter, texture, color, and the dimensions and morphology of hyphae and conidia (Ainsworth et al., 1973).

DNA extraction, ITS-rDNA amplification and sequence analysis

Total genomic DNA of the fungal isolates 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 ITS-rDNA amplification were referenced by our previous reports (Xu et al., 2008; Li et al., 2008). For identification, the polymerase chain reaction (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 sequences were run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and they were submitted to GenBank where the accession numbers were obtained.

Mycelial suspension culture and *n*-butanol 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 cultures of eleven endophytic fungal isolates purified on PDA. All flasks were incubated at 150 rpm on a rotary shaker at 25°C for 15 days. After suspension culture, the culture broth was filtrated in vacuum to afford the filtrate and mycelia. The filtrates were extracted with an equal volume of *n*-butanol for three times. The mycelia were lyophilized and powdered, followed by extracting with ultrasound in *n*-butanol for three times. The *n*-butanol solutions were concentrated in vacuum at 50°C to obtain mycelia and filtrate extracts, respectively.

Detection of antimicrobial activity of the extracts

Thin layer chromatography (TLC)-bioautography assay of the samples was carried out according to the method of Zhao et al. (2008). Four bacterial strains *Bacillus subtilis* ATCC 11562, *Escherichia coli* ATCC 25922, *Pseudomonas lachrymans* ATCC 11921, and *Xanthomonas vesicatoria* ATCC 11633 were selected for antibacterial assay. After the TLC plate with the test bacterium was incubated at 28°C for 12 h, the color reagent was sprayed with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA), and incubated for another 2 h. The presence of biological activity was determined by the formation of well-defined inhibition zones that was made visible by spraying with MTT that was converted to a formazan dye by the living microorganism (Bernas and Dobrucki, 2000). Antibacterial activity was detected as white inhibition zones against a purple background, and the Rf value of the antibacterial area was determined (Xu et al., 2010). Rf = D_1/D_2 where D_1 is the distance between the antimicrobial area and initial sample point, and D_2 is the distance between the developing solvent front and initial sample point on a TLC plate. Four phytopathogenic fungal strains *Botrytis cinerea*, *Botryosphaeria dothidea*, *Fusarium oxysporum* f.sp. *niveum*, and *Magnaporthe oryzae* were selected for antifungal assay. After the TLC plate with the test fungus was incubated at 25°C for 4 to 7 days, the inhibition zone of the mycelia growth was visible, and the Rf value of the antifungal area was determined without MTT treatment. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Identification of the endophytic fungi

A total of forty-three endophytic fungal isolates were separated from the twigs and barks of the poplar hybrid 'Neva' of *P. deltoides* Marsh × *P. nigra* L. According to their morphological features, 11 distinct fungal isolates were selected for further taxonomical identification. They were identical to the members of different genera, that is, *Alternaria*, *Aureobasidium*, *Botryosphaeria*, *Hyalodendriella*, *Mycosphaerella*, *Peyronellaea*, *Phoma* and *Valsa* according to the results of the macro and microscopic identification (Table 1), indicating the diversity of the fungi associated with the poplar hybrid 'Neva'. The fungi of genera *Alternaria* were the main isolates including three groups which CF were 18.6% (Ponipodef01), 9.3% (Ponipodef04) and 14.0% (Ponipodef07), respectively.

The ITS1-5.8S-ITS4 partial sequences of 11

Table 1. Colonization frequency (CF) of the endophytic fungi and their closest relatives based on the data from BLAST analysis and morphological identification.

Fungal Isolate	CF (%)	GenBank accession number	Closest related species	Similarity (%)	Macro and microscopic identification
Ponipodef01	18.6	HQ731637	*Alternaria* sp.	99	*Alternaria* sp.
Ponipodef02	14.0	HQ731638	*Phoma* sp.	99	*Phoma* sp.
Ponipodef03	2.3	HQ731639	*Botryosphaeria berengeriana*	99	*Botryosphaeria* sp.
Ponipodef04	9.3	HQ731640	*Alternaria alternata*	100	*Alternaria* sp.
Ponipodef05	2.3	HQ731641	*Valsa sordida*	99	*Valsa* sp.
Ponipodef06	4.7	HQ731642	*Mycosphaerella aleuritidis*	97	*Mycosphaerella* sp.
Ponipodef07	14.0	HQ731643	*Alternaria tenuissima*	100	*Alternaria* sp.
Ponipodef08	2.3	HQ731644	*Peyronellaea glomerata*	100	*Peyronellaea* sp.
Ponipodef09	4.7	HQ731645	*Botryosphaeria dothidea*	99	*Botryosphaeria* sp.
Ponipodef12	11.6	HQ731647	*Hyalodendriella* sp.	97	*Hyalodendriella* sp.
Ponipodef15	7.0	HQ731650	*Aureobasidium pullulans*	99	*Aureobasidium* sp.

representative isolates were submitted to the GenBank to obtain their accession numbers, and the closest related species were got by BLAST analysis. The results showed that all the sequences had more than 97% similarity with the species in GenBank. The molecular characters of the endopytic fungi were basically coincident with their morphology ones (Table 1).

Some species of *Botryosphaeria* and *Valsa* were previously reported to be the fungal pathogens of *Populus* species (Wang et al., 1981; Zhou et al., 2008). Whether the isolates Ponipodef05 (*Valsa* sp.), Ponipodef03 (*Botryosphaeria* sp.), and Ponipodef09 (*Botryosphaeria* sp.) separated from the healthy tissues in this study are pathogenic or non-pathogenic needs further verification. To our knowledge, Ponipodef06 (*Mycosphaerella* sp.) and Ponipodef12 (*Hyalodendriella* sp.) were isolated from *Populus* species for the first time. In this study, only eleven distinct fungal endophytes were identified from the healthy twigs and barks of the poplar hybrid 'Neva'. It is possible that we have adopted a strict surface sterilization, and many endophytic fungi are uncultured. Though there were some reports about the endophytic fungi from other species or hybrids of *Populus* species such as *Populus tremula* (Santamaria and Diez, 2005; Albrectsen et al., 2010), *Populus tremuloides* (Hutchison, 1999), *Populus trichocarpa* (Xin et al., 2009), and a hybrid of *P. trichocarpa* × *P. deltoides* (Xin et al., 2009), this is the first report on the endophytic fungi associated with the poplar hybrid 'Neva'.

Detection of antimicrobial activity

Antimicrobial activities of the mycelia and filtrate extracts were showed in Tables 2 and 3. Rf values of the antimicrobial areas can indicate the relative polarity of the active components in the samples, and the diameters can indicate the relative antimicrobial activity of the components. Most of the extracts displayed their antibacterial activity except the filtrate extract of isolate Ponipodef15. The extracts of Ponipodef04, Ponipodef05 and Ponipodef12 were found to have their stronger antibacterial activity than other extracts. Generally, the antibacterial activity of each extract was stronger than that of its antifungal activity. Some of the extracts (e.g. Ponipodef02 and Ponipodef07) did not show any inhibitory zones on test fungi. The extracts of Ponipodef05 and Ponifodef12 were found to show their strong antibacterial and antifungal activities. This indicates that both antifungal and antibacterial components exist in these two isolates.

In summary, we first reported the endophytic fungi from the poplar hybrid 'Neva' of *P. deltoides* Marsh × *P. nigra* L. and the detection of the antimicrobial activities of their extracts, some of which (e.g. Ponipodef05 and Ponipodef12) displayed strong antibacterial and antifungal activities. The endophytic fungi from the poplar hybrid 'Neva' could be an appropriate source to produce antimicrobial agents. Isolation of the antimicrobial compounds from these fungi is now in progress. The results from this study also provide additional data for realizing the physiological and ecological roles of the poplar endophytic fungi.

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Table 2. Antibacterial activity of the crude extracts from the endophytic fungi against different bacteria by TLC-bioautography-MTT test.

Fungal isolate	M/F	Rf value of the antibacterial area (Diameter of the antibacterial area)			
		Bacillus subtilis	***Escherichia coli***	***Pseudomonas lachrymans***	***Xanthomonas vesicatoria***
Ponipodef01	M	0-0.90(++)	0-0.92(++)	0-0.92(++)	0.22-0.51(+)
	F	0.32-0.43(+)	0-0.22(++)	-	0.33(+)
Ponipodef02	M	0-0.22(+)	0-0.22(+)	0-0.21(+)	0-0.33(+)
	F	0-0.1(+)	0-0.1(+)	0-0.1(+)	0-0.1(+)
Ponipodef03	M	0-0.33(+)	0-0.23(+)	0.13(+)	0-0.25(+)
	F	0.27-0.52(+)	0-0.6(++)	0-0.20(+)	-
Ponipodef04	M	0-0.42(++)	0-0.31(++)	0-0.37(+)	0-0.22(+)
	F	0-0.7(+)	0-0.58(++)	0-0.13(+)	0(+)
Ponipodef05	M	0-0.67(++)	0-0.90(++)	0-0.55(++)	0-0.72(++)
	F	0-0.63(++)	0-0.44(++)	0-0.67(++)	0-0.67(++)
Ponipodef06	M	0-0.45(++)	0-0.23(++)	0-0.33(+)	0-0.40(+)
	F	0(+)	0(+)	-	0-0.13(+)
Ponipodef07	M	0-0.17(+)	0.10-0.23(+)	0.10-0.27(+)	0.25-0.38(+)
	F	0-0.48(+)	0(+)	0.27(+)	0-0.35(+)
Ponipodef08	M	0-0.13(+)	0-0.17(+)	0-0.17(+)	0-0.18(+)
	F	0-0.67(++)	0-0.23(++)	0-0.20(+)	0-0.12(+)
Ponipodef09	M	0-0.40(+)	0(+)	0(+)	0-0.50(+)
	F	0-0.70(++)	0-0.23(++)	0-0.45(++)	0-0.35(+)
Ponipodef12	M	0-0.8(+++)	0-0.65(++)	0-0.45(++)	0-0.43(+)
	F	0-0.10(+)	0-0.20(++)	0-0.23(+)	0-0.30(++)
Ponipodef15	M	0-0.1(+)	0-0.1(+)	0-0.1(+)	0-0.12(+)
	F	-	-	-	-

Note: M, mycelia *n*-butanol extract; F, filtrate *n*-butanol extract; Developing solvent system in TLC was petroleum ether-acetone (15:8, v/v); -, no antimicrobial activity was observed; +, the diameter of the antimicrobial activity area was 0-5 mm; ++, the diameter of the antimicrobial activity area was 5-10 mm; +++, the diameter of the antimicrobial activity area was more than 10 mm; The positive control was streptomycin sulfate which was only sampled on the TLC plate and showed antibacterial activity.

Table 3. Antifungal activity of the crude extracts from the endophytic fungi against different phytopathogenic fungi by TLC-bioautography test.

Fungal isolate	M/F	Rf value of the antifungal area (Diameter of the antifungal area)			
		Botrytis cinerea	***Botryosphaeria dothidea***	***Fusarium oxysporum* f.sp. *niveum***	***Magnaporthe oryzae***
Ponipodef01	M	-	0-0.27(+)	-	0(+)
	F	-	0-0.08(+)	-	-
Ponipodef02	M	-	-	-	-
	F	-	-	-	-
Ponipodef03	M	-	0(+)	0-0.42(+)	0(+)
	F	-	-	-	-

Table 3. Contd.

Ponipodef04	M	-	0-0.17(+)	-	0(+)
	F	0(+)	-	-	-
Ponipodef05	M	0-0.25(++)	0-0.65(+)	0-0.58(++)	0-0.65(++)
	F	0(+)	0-0.12(+)	0-0.33(+)	0(+)
Ponipodef06	M	-	-	-	0(+)
	F	0(+)	-	-	0(+)
Ponipodef07	M	-	-	-	-
	F	-	-	-	-
Ponipodef08	M	-	-	-	-
	F	-	-	0(+)	-
Ponipodef09	M	-	0(+)	-	-
	F	-	-	-	-
Ponipodef12	M	0-0.53(++)	0-0.57(++)	0-0.25(++)	0-0.65(+)
	F	0(+)	0-0.48(++)	0-0.15(+)	0-0.32(+)
Ponipodef15	M	-	-	-	-
	F	-	-	-	-

Note: The positive control was carbendazim which was only sampled on the TLC plate and showed antifungal activity. Other notes are the same as those in Table 2.

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