SUPPLEMENTARY MATERIAL

Antimicrobial activity of saponins produced by two novel endophytic fungi from *Panax notoginseng*

Zhaoxia Jin*, Lin Gao, Lin Zhang, Tianyi Liu, Fang Yu, Zongshen Zhang, Qiong Guo and Biying Wang

School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China

Abstract

Endophytes in plants may be co-producer of the bioactive compounds of their hosts. We conducted a study to bioprospect for saponin-producing endophytic fungi from *Panax notoginseng* and evaluate the antimicrobial activity of saponins. Two novel fungal endophytes, *Fusarium* sp. PN8 and *Aspergillus* sp. PN17, were isolated from traditional Chinese medicinal herb *P. notoginseng*. After 8 days fermentation, the total saponins produced in the culture broth of PN8 and PN17 were 1.061 mg mL^{-1} and 0.583 mg mL^{-1}, respectively. The saponin extracts exhibited moderate to high (inhibition zone diameter 15.7-28.4 mm, MIC 1.6-12.5 mg mL^{-1}) antimicrobial activity against pathogens tested. Further analysis showed that triterpenoid saponins produced by *Fusarium* PN8 were Rb_1, Rd and 20(S)-Rg_3, while *Aspergillus* PN17 had the ability to synthesize ginsenoside Re, Rd and 20(S)-Rg_3. The isolated endophytes may be used as potentially sources for microbial production of plant secondary metabolites and for antimicrobial agents.

Keywords: Endophytes; *P. notoginseng*; saponins; *Fusarium*; *Aspergillus*; antimicrobial activity

Experimental

Isolation of endophytic fungi from P. notoginseng

The endophytes were isolated from *P. notoginseng* using a modified method described by Miller et al. (2012). Plant materials were carefully washed with running water and then surface sterilized successively by immersing in 70% ethanol for 1 min, 1% mercuric chloride for 5 min, followed by rinsing thrice in sterile water. The tissues samples were placed on nutritive agar to confirm the surface was aseptic. Small segments (1 cm) of inner tissue of sterilized seeds and roots were spread on potato dextrose agar (PDA) medium plates and incubated at 28°C for 2 days. The colonies emerged around the segments were transferred to new PDA plates for sub-culturing.

Identification and characterization of fungal isolates

The isolated endophytes were identified by morphological characteristics and ITS sequence analyses. Total genomic DNA was extracted by using the protocol described previously (Jin et al. 2014). The ITS gene sequence was amplified under standard PCR conditions with the universal primers (ITS1: 5′-TCC GTA GGT GAA CCT GCG G-3′) and (ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′) (Liu et al. 2008). Purified PCR products were sequenced and aligned with sequences available in GenBank by using the BLASTn similarity searches. A phylogenetic tree was constructed with the neighbor-joining algorithm and the distance values were calculated using software program MEGA version 4.1 (Kumar et al. 2008).

The ITS gene sequences of *Fusarium* strain PN8 and *Aspergillus* strain PN17 have been deposited in the GenBank under accession no. KX179640 and KX179644.

Extraction and determination of total saponins

Each endophyte was inoculated into 100 mL of PDA medium and cultured at 28°C for 8 days with shaking at 180 rpm. The collected mycelia were re-suspended in sterile water and the cells were broken by an ultrasonic disruptor for 10 min. The suspension was then centrifuged at 7024 g for 10 min and the supernatant was collected as mycelia extracts. A 30 mL aliquot of broth or mycelia extracts were added with ethanol to make the final concentration of 80%. The mixed solution

stood for 24 h and was centrifuged at 7024 g for 15 min. The supernatant was collected and evaporated to dryness in a water bath at 60ºC, and then 10 mL of water was added. After removing pigment in the samples with petroleum benzin, saponins were extracted with same volume of water saturated n-butanol from water phase. The total saponins concentration of extracts was determined using by the vanillin-sulfuric acid method (Chen et al. 2010), and uninoculated sterile broths were used as controls.

Determination of antimicrobial activity of saponin extracts

In vitro antimicrobial activity of endophytes against five pathogenic bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC25923, *Sarcina lutea* ATCC 9341) and two yeast (*Candida albicans* ATCC 90028, *Saccharomyces cerevisiae* ATCC Y139) were evaluated by the paper disc method (Gopiesh khanna et al. 2008)**.** Briefly, aliquots of 100 µl each microbial suspension containing approximately 10^6 CFU mL^{-1} of cell were inoculated on Muller Hinton agar (MHA) (for bacteria) and Sabouraud Dextrose Agar (for yeast) plates. Crude saponin extracts of endophytes were sterilized using 0.2 µm membrane filter. Sterilized paper discs (Φ = 6 mm) were impregnated with 30 µL of the extracts and placed on the inoculated plates. The diameters of inhibition zones were measured after 24 h of incubation at 37 ºC for bacteria or 72 h incubation at 28 ºC for yeasts. All experiments were performed in triplicate. Total saponins standard and Gentamicin were included as positive control while water saturated n-butanol used as the negative control, respectively.

Broth dilution methods and different concentrations of saponin extracts (0.4 - 25 mg mL^{-1}) were used to determine minimal inhibitory concentration (MIC) according to Gopiesh khanna et al. 2008. After incubation under optimal conditions, the minimum concentration of saponin extracts that inhibited visible growth of each pathogen tested was recorded as the MIC.

Analysis of saponins constituents by HPLC and HPLC-MS

The constituents of saponins were determined by HPLC (Waters2695, USA) equipped with Unitary-C18 reversed-phase column (250 × 4.6 mm×5 µm). The mobile phase

consisted of acetonitrile and water, and the elution gradient of acetonitrile volume fraction was as follows: 0-20 min, 20%; 21-30 min, from 20% to 32%; 31-40 min, from 32% to 43%; 41-70 min, from 43% to 100%. The flow rate was 0.6 mL min^{-1}, and the injection volume was 10 μL. The eluted peaks were detected by the UV detector with a wavelength of 203 nm.

HPLC–MS analyses of ginsenosides were conducted on an Agilent 1200 Series HPLC equipped with 6130 single quadrupole mass spectrometer. The HPLC conditions were as described above. The mass spectra were recorded with negative ion ESI over the range 120-1800 m/z. Drying gas was nitrogen (flow-rate of 10 L min^{-1}, 350℃), and the source CID voltage and the sheath gas pressure was set at 50 v and 40 psi, respectively.

Table S1. In vitro antimicrobial activity of saponin extracts of endophytic fungi against various

Train	*F. oxysporum* PN8		*A. niger* PN17		Total saponins		Gentamicin (300 μg mL^{-1})		Amphotericin (300 μg mL^{-1})	
	Diameter of inhibition zone（mm）	MIC (mg mL^{-1})	Diameter of inhibition zone（mm）	MIC (mg mL^{-1})	Diameter of inhibition zone(mm)	MIC (mg mL^{-1})	Diameter of inhibition zone（mm）	MIC (mg mL^{-1})	Diameter of inhibition zone（mm）	MIC (mg mL^{-1})
Escherichia coli ATCC 25922	23.1±3.1	3.2	25.3±3.5	3.2	30	0.1	37	0.001	NT	NT
Pseudomonas aeruginosa ATCC 27853	15.7±2.8	12.5	16.1±2.5	12.5	32	0.2	40	0.0005	NT	NT
Staphylococcus aureus ATCC 25923	26.2±1.6	3.2	28.4±2.1	1.6	29	0.1	35	0.001	NT	NT
Bacillus subtilis ATCC 6633	16.5±2.1	12.5	16.9±1.9	12.5	23	0.5	38	0.001	NT	NT
Sarcina lutea ATCC 9341	27.8±1.2	1.6	24.6±1.8	1.6	32	0.5	32	0.002	NT	NT
Saccharomyces cerevisiae ATCC Y139	18.4±3.4	6.3	16.8±3.3	6.3	24	0.5	NT	NT	30	0.0004
Candida albicans ATCC 90028	22.6±1.6	3.2	24.1±1.5	3.2	27	0.5	NT	NT	27	0.0008

NT: not tested

Table S2. Ginsenosides and total saponins contents in culture broth of two endophytes

Ginsenoside	Type	$[M-H]^-$	$[M+Cl]^-$	$[M+NO_3]^-$
Rb1	20(s)-protopanaxadiol	1107.6	1143.6	1170.6
Rd	20(s)-protopanaxadiol	945.6	981.6	1008.6
Re	20(s)-protopanaxadiol	945.6	981.6	1008.6
Rg3	20(s)-protopanaxadiol	783.5	819.5	846.5

Table S3. Major ions (m/z) observed in the negtive ion HPLC-ESI-MS spectra of the ginsenoside standards

Ginsenosides	Time (min)	*Fusarium* sp. PN8 (mg mL^{-1})	*Aspergillus* sp. PN17 (mg mL^{-1})
Re	32.193	ND	0.33
Rb1	41.746	0.13	ND
Rd	45.322	0.64	0.24
20(s)-Rg3	51.911	0.19	0.13
Total content		0.96	0.70

Table S4. The identification of ginsenosides in endophytes *F. oxysporum* PN8 by HPLC-ESI-MS

Ginsenoside	Intact molecular ion	$[M-H]^-$	$[M+Cl]^-$	$[M+NO_3]^-$
Rb1	1108.6	1107.6	1143.6	1170.6
Rd	946.6	945.6	981.6	1008.6
Rg3	784.5	783.5	819.5	846.5

Table S5. The identification of ginsenosides in endophytes *A. niger* PN17 by HPLC-ESI-MS

Ginsenoside	Intact molecular ion	$[M-H]^-$	$[M+Cl]^-$	$[M+NO_3]^-$
Rd	946.6	945.6	981.6	1008.6
Re	946.6	945.6	981.6	1008.6
Rg3	784.5	783.5	819.5	846.5

Figures

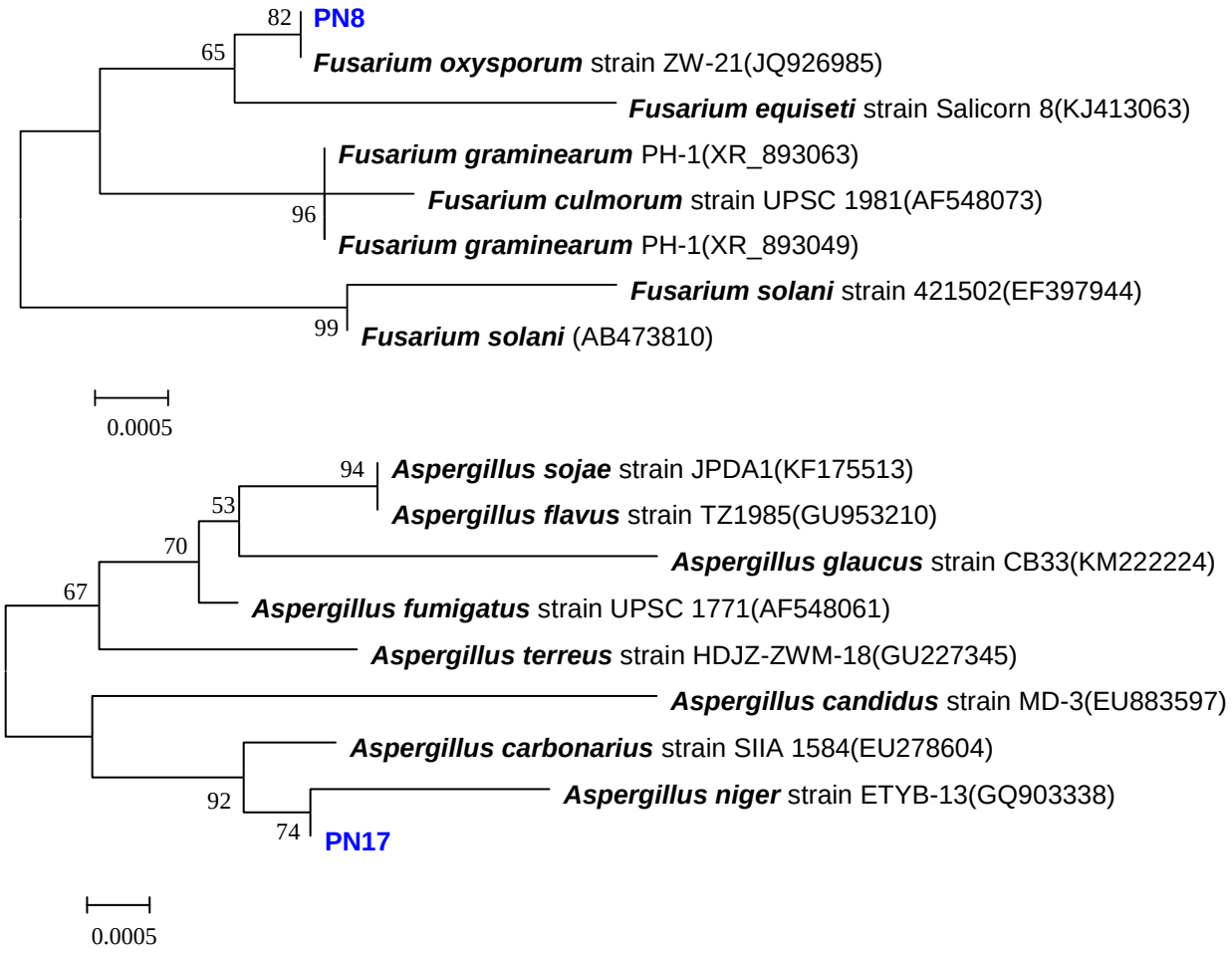


Figure S1. Neighbour-joining phylogenetic tree based on ITS gene sequences showing the position of the two isolates within the genus *Fusarium* and *Aspergillus*, respectively. Bootstrap percentages (>50%) after 1000 resamplings is shown. Bar = 0.5% sequence divergence.

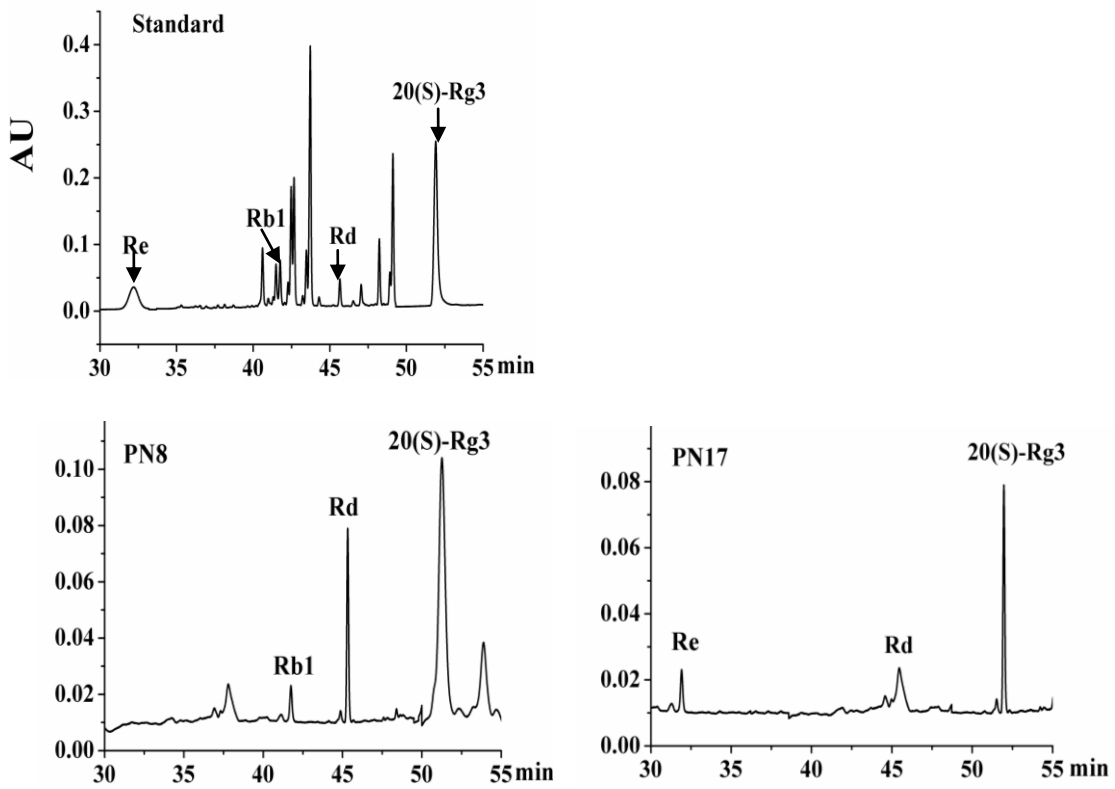
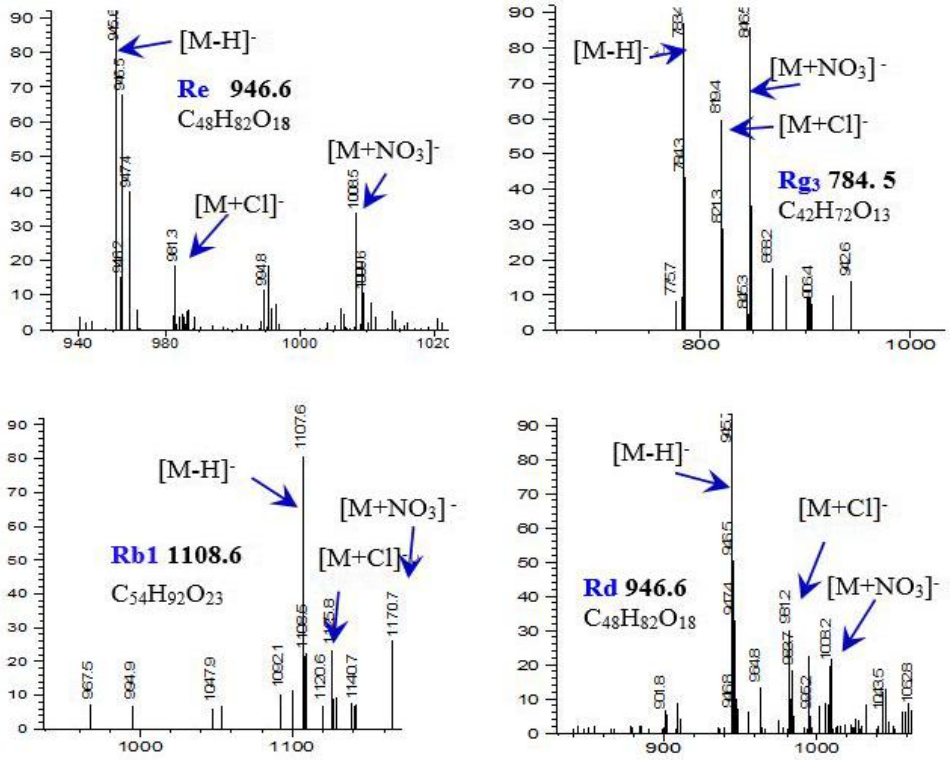
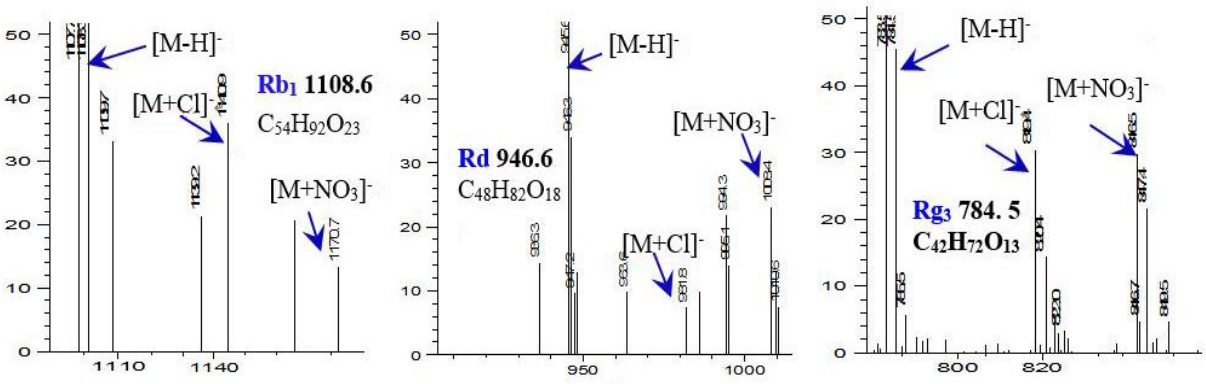


Figure S2. HPLC chromatograms of saponins production of endophytic fungi *F. oxysporum* PN8 and *A. niger* PN17. Standard ginsenoside Re, Rb_1, Rd and 20(S)-Rg_3 with retention time of 32.193min, 41.755 min, 45.652 min and 51.911 min respectively.

Standard



***F. oxysporum* PN8**



***A. niger* PN17**

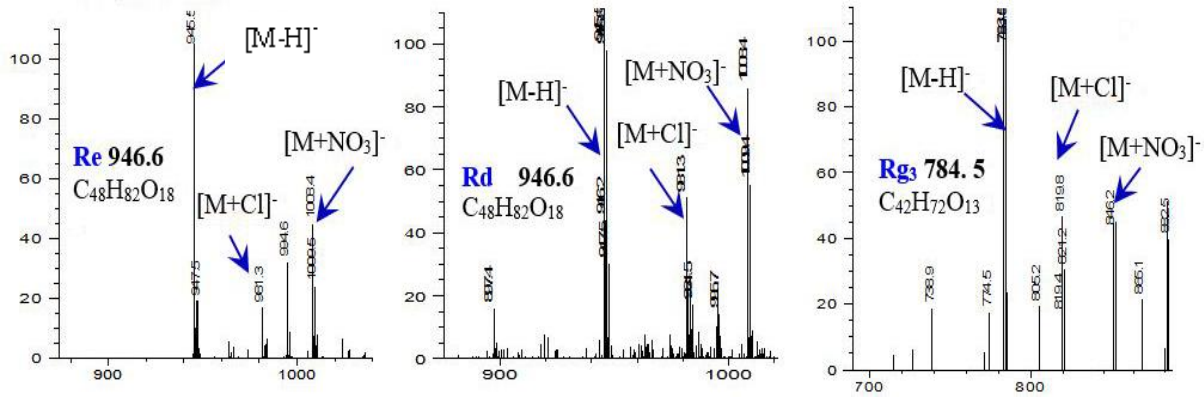


Figure S3. HPLC-MS negative ion spectra of ginsenosides detected in endophytes *F. oxysporum* PN8 and *A. niger* PN17.

References

Chen YF, Yang CH, Chang MS, Ciou YP, Huang YC. 2010. Foam properties and detergent abilities of the saponins from *Camellia oleifera*. Int. J Mol Sci. 11:4417-4425.

Gopiesh khanna V, Kannabiran K. 2008. Antimicrobial activity of saponin fractions of the leaves of *Gymnema sylvestre* and *Eclipta prostrate*. World J Microbiol Biotechnol. 24:2737–2740.

Jin ZX, Guo Q, Zhang ZS, Yan TS. 2014. Biodegradation of type II pyrethroids and major degraded products by a newly isolated *Acinetobacter* sp. strain JN8. Can J Microbiol. 60:541-545.

Kumar S, Nei M, Dudley J, Tamura K. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform. 9:1-8.

Liu XL, Dong MS, Chen XH, Jiang M, Lv X, Zhou JZ. 2008. Antimicrobial activity of an endophytic *Xylaria* sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin. Appl Microbiol Biotechnol. 78:241–247.

Miller KI, Qing C, Sze DMY, Roufogalis BD, Neilan BA. 2012. Culturable endophytes of medicinal plants and the genetic basis for their bioactivity. Microb Ecol. 64:431-449.