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Endophyte Fungal Isolates from *Podophyllum peltatum* Produce Podophyllotoxin

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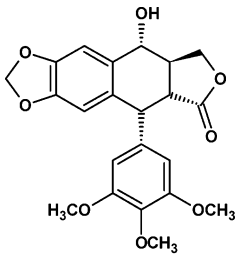
Abstract: The lignan podophyllotoxin (**1**) is highly valued as the precursor to clinically useful anticancer drugs. Substantial drug development of this compound class continues, including potential new use for inflammatory disease. We have isolated two endophyte fungi, both strains of *Phialocephala fortinii*, from rhizomes of the plant *Podophyllum peltatum*. The fungi were identified through DNA sequencing and morphology. Both strains of fungi are slow-growing and produce **1** at low but measurable amounts in broth culture. The compound was confirmed through matching HPLC retention times, absorption spectra, and MS data to authentic **1**. The yield of **1** has ranged from 0.5 to 189 μg/L in 4 weeks of culture. These fungi have implications for the sustained production of **1** independent of wild populations of the source plants.

Podophyllotoxin (**1**), an aryltetralin lignan, is a valuable natural product as a precursor to three anticancer drugs, etoposide, teniposide, and etoposide phosphate.[1] Destruction of wild populations of the primary source plant (*Podophyllum emodi* Wall. ex Royle)[2] and difficulties of total synthesis of the compound[3] have led many laboratories to search for alternative sources. Endophyte fungi are widespread and perhaps ubiquitous in their occurrence in higher plants.[4] Ever since the several reports by Strobel's group of endophyte fungi from a variety of yew species that produce taxol, (e.g., ref 5), there has been interest in the biosynthetic capabilities of endophytes for a variety of natural products. Fungal endophytes have begun to be recognized as a rich and diverse source of natural products.[6] Recently, both camptothecin[7] and podophyllotoxin (**1**)[8] have been reported to be produced by endophytes from *Nothapodytes foetida* and *P. emodi*, respectively. We report here the isolation of two strains (PPE5 and PPE7) of *Phialocephala fortinii* Wang & Wilcox (Leotiomycetes, Ascomycota) from wild collections of *Podophyllum peltatum* L. (Berberidaceae) that produce measurable amounts of **1** in culture.[9]



1

Hartwell[10] first isolated **1** during observations of treatment of venereal warts with *P. emodi* resin. The compound was subsequently confirmed to have antineoplastic activity through inhibition of tubulin polymerization and disruption of mitosis during metaphase.[11] Modifications of the molecule to increase solubility and reduce gastric toxicity led to semisynthetic derivative compounds commercialized as etoposide and teniposide. These compounds exhibited the desired properties but were also found to have a different mechanism of action, topoisomerase II inhibition.[11] The inability of the cells to properly repair DNA due to this enzyme inhibition also disrupts the cell cycle. The phosphate derivative, Etopophos, targets the toxic compound more specifically to cancer cells due to their higher alkaline phosphatase activity.[1] This activity releases etoposide *in cellulo* from the relatively nontoxic Etopophos. Other derivatives are being developed for cancer, e.g., NK 611[11] and GL-331,[12] and for rheumatoid arthitis, Reumacon (CPH 82).[13]

Relatively high amounts of **1** are found in the leaf and rhizome tissues of *Podophyllum* spp., occurring at levels of over 5% of the dry matter.[1] Canel et al.[14] determined that buffered wetting of dry leaf tissue releases substantially more of the compound through release of the aglycon from the bound glycoside form due to the action of native glycosidases. This group has also determined that different clones vary in the amount of **1** and the biosynthetic peltatin precursors,[15] which is not entirely explained by environmental conditions. This work has extended the supply of podophyllotoxin

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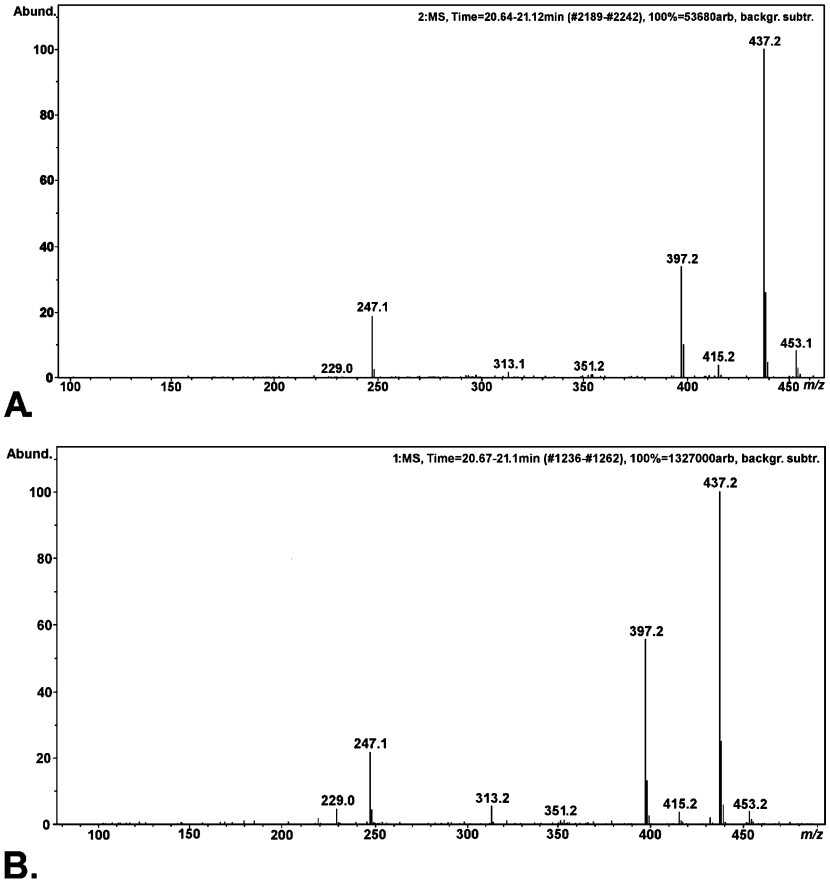

Figure 1. ESIMS of podophyllotoxin (**1**) derived from the *P. fortinii* PPE 7 culture medium (A) and commercial **1** (B).

(**1**) by switching to the North American mayapple (*P. peltatum*) as an alternative source. However, the growth rate and life history of this plant almost certainly ensure that decline in populations will occur with increased harvesting.[16] The inability to synthesize **1** in an economic scale has led to further investigation of other strategies to increase availability of the drug precursor. These approaches include cell culture of *Linum* spp.,[17] transformation of *E. coli* with lignan pathway biosynthetic genes from *Forsythia* and *Thuja*,[18] and tissue culture approaches using *Anthriscus.*[19] Substantial work continues to develop additional derivatives of **1** for the treatment of neoplastic and inflammatory diseases. Identification of a cultivable microorganism that produces **1** could ease substantially the pressure on wild plant populations, ensure supply of this valuable natural product, release production from the vagaries of climate and politics, and provide scalability of production to meet future drug development and delivery needs.[20]

Of the 299 explants obtained from *P. peltatum* plants, there were 215 fungal isolates, and 18 were determined to be unique morphologically. These were designated as putative endophytes (no matching isolates from unsterilized explants). Two of these were found to have a peak matching the retention time and absorption spectrum (Figure S1, Supporting Information) of authentic **1** in the culture medium at the end of the culture period. The *P. fortinii* endophytes grow slowly in culture. Typically, 8.5 g of biomass (dry weight) is produced in 1 L of malt extract broth (MB) or yeast-malt broth (YMB) in 4 weeks of culture. None of the cultured fungal biomass Soxhlet extractions yielded peaks matching **1**. Each putative endophyte was subcultured to ensure an axenic culture; each fungal culture has been continuously maintained since the original isolation, with transfer to fresh medium every 4−6 weeks. This ensures that the **1** seen was not carry-over from the explant. Subsequent subculturing and reanalysis for **1** have confirmed this conclusion. The two isolates containing **1** in the culture medium were confirmed by LC-MS analysis of the peaks. The ESIMS of the fungal compound had peaks characteristic of plant-derived commercial podophyllotoxin (**1**) (from *P. emodi*, Sigma) {$[M + H]^+$, *m*/*z* 415.2 (2); $[M + Na]^+$, *m*/*z* 437.2 (100); $[M + K]^+$, *m*/*z* 453.1 (8); $[M - OH]^+$, *m*/*z* 397.2 (34); $[M - OH - CO_2]^+$ (ring A), *m*/*z* 351.2 (1); $[M - OH - C_2O_2H_2$ (ring A) − COH (ring D)$]^+$, *m*/*z* 313.1 (2); $[M - C_6H_2(OCH_3)_3$ (ring E)$]^+$, *m*/*z* 247.1 (18), $[M - OH - C_6H_2(OCH_3)_3$ (ring E)$]^+$, *m*/*z* 229.0 (0.4)}. The area under the curve of a podophyllotoxin (**1**) peak in HPLC was compared to a standard curve of authentic **1** to determine its production by a culture. The initial results showed 20 μg/L of **1** in 4 weeks of culture. Subsequent cultures have yielded **1** over a range of 0.5 to 189 μg/L in 4 weeks of culture.

Both the fungal biomass and the culture medium from a grown culture were assessed for podophyllotoxin (**1**). None of the biomass collections yielded any trace of **1** or its glycoside. Further studies in which the biomass was refluxed with 1.0 N HCl prior to extraction with EtOH or EtOAc also did not yield **1**. Acid hydrolysis breaks the glycoside bond to yield free **1**.[14] Culture media from two of the endophyte fungi, designated strains PPE5 and PPE7, gave peaks that matched retention time (ca. 32.6 min) and absorption spectra (λ_{max} 205−206, 245, 290 nm) of authentic **1** (Figure S1, Supporting Information). These peaks were further analyzed by LC-MS. The mass spectrum matched that of **1** (Figure 1).

The two podophyllotoxin-producing fungal isolates were identified on the basis of the sequence of the D2 region of the 28S rDNA. The sequences were compared to GenBank and the MicroSeq databases. The sequences most closely matched entries corresponding to *P. fortinii*, with a difference of 0.00% for strain PPE5 and 5.03% for strain PPE7 compared to the MicroSeq database sequence. Sequence identities were confirmed using morphology of the fungi in culture. *P. fortinii* is a known endophyte species complex occurring in a variety of plant species.[4] Its ability to produce **1** or any other secondary metabolite has not been tested

previously. A patent application has been filed to cover the production of **1** with the endophytic fungi.[21] The DNA sequences (Figure S2, Supporting Information) obtained have been deposited into GenBank as accessions DQ485455 and DQ485456. The endophytic fungi have been deposited at ATCC as *P. fortinii* with accession numbers ATCC PTA-5208 and ATCC PTA-5209. The *P. fortinii* strains obtained in this study are genetically distinct from *Trametes hirsuta*, an endophyte from *P. emodi* reported to produce **1** in culture[8] (Figure S3, Supporting Information).

We examined the biological activity of the **1**-containing extract through the use of the brine shrimp lethality assay. Authentic **1** gives an LD_{50} in the range of 2−3 μg/mL in this assay. When the extracts putatively containing **1** were tested in the assay, the results were as expected for an equivalent amount of **1** as was estimated in the extract.

The discovery of fungal endophytes that produce **1** has significant biological and commercial implications. Commercially, the fungal culture can be scaled to provide adequate **1** production to satisfy new drug development and patient treatment needs. This production reduces the need to harvest wild populations of the source plants, preserving these species for the future. Biologically, the production of a toxic metabolite by fungal endophytes raises some interesting questions. It is not yet known whether the origin of the biosynthetic pathways of metabolites such as **1**, paclitaxel, or camptothecin originated in a plant or microbial organism. Also, it is not known whether horizontal transfer of the genes that support the biosynthesis is a common occurrence or occurs only under specialized conditions. Although the actual source organism (fungus or plant) of paclitaxel and camptothecin remains in question due to this kind of research, the low production of **1** in culture by endophytes from *P. peltatum* does not lend strong support to the endophyte as the source of the high content in the leaf and rhizome tissues. However, metabolic regulation of an endophytic fungus in axenic culture is likely to be substantially different from that which occurs in the host plant.[20] It is highly probable that a genetic regulatory system similar to the *Aspergillus* LaeA signal transduction mechanism[22] is involved in regulation of natural product biosynthesis in this and other endophytic fungi responding to plant metabolic and other biotic and abiotic cues. The role of the fungus in the production of **1** in *P. peltatum* and regulation of that production needs further investigation. In addition to optimization studies to increase the production by the cultured fungal endophytes, we are undertaking analysis in an effort to answer some of the genetic questions that remain.

Experimental Section

General Experimental Procedures. Putative **1** peaks were subjected to LC-MS analysis using an Agilent 1100 series HPLC, PDA detector, HP/Bruker ion trap mass spectrometer. An Alltech Hypersil BDS column was used to analyze 25 μL injections using a 5−95% acetonitrile/water gradient over 40 min (1 mL/min). Peaks matching authentic **1** at 20.9−21.0 min were analyzed using a scan range of 50−850 Da and an accumulation time of 850 μs. Mass spectrometric data were collected and processed using BrukerData Analysis software. Absorption spectra data for peaks were collected either on the Agilent PDA listed above or on a Dionex UVD340U PDA detector.

Isolation and Culture of Endophytic Fungi. As part of an effort to identify endophyte fungi that produce **1**, wild specimens of *P. peltatum* were collected from a natural population near Newark, DE, in the spring of 1999. A specimen from the population has been deposited in the herbarium of the University of the Sciences in Philadelphia (PHL) (accession no. Eyberger01). In addition, a potted specimen was obtained at a local nursery. The plants were removed from the soil, washed in deionized (DI) water, followed by 1% LiquiNox to remove soil contamination, and divided into leaf, petiole, rhizome, and root portions. These parts were surface sterilized by placing the separated plant parts into 95% EtOH for 1 min, 20% commercial bleach for 3 min, fresh 95% EtOH for 0.5 min, and a final rinse with sterile, filtered DI water. Each sterilized plant part was aseptically sectioned into 1 cm long segments or 1 × 1 cm squares, as appropriate. Non-surface-sterilized plant parts were prepared identically in parallel for the isolation of surface-contaminating fungi.

Table 1. Mean Recovery[a] (%) of **1** from Water and Culture Medium (YMB) by C_{18} Solid-Phase Extraction.

	matrix	
concentration (g/L)	water	YMB
50[b]	97.1	78.0
200	94.8	64.9

[a] Quantitation equation: $y = 80.9x + 409.6$; $r = 0.9986$; $n = 5$ [b] Limit of quantitation was 0.5 μg/L based on a signal-to-noise ratio of 10.

The prepared plant segments and squares were plated onto 1% malt extract agar (Difco 0112-17-6) with or without 0.1% streptomycin sulfate to inhibit the growth of bacteria. The plates were incubated at 20−23 °C in the dark, 20−23 °C in the light (GE F15T8/PL/AQ/WS fluorescent lamps), or 4 °C in the dark. A total of 169 explant cultures were prepared from wild collected plants and 130 explants from the potted plants. Fungal isolates were subcultured as they appeared over 4−6 weeks by transferring small cubes of agar containing hyphal tips to fresh agar. Isolates that occurred on both surface-sterilized and -unsterilized segments were not maintained, as they were considered to be surface contaminants. Fungal isolates that were unique to the surface-sterilized plant material were subcultured onto agar to ensure an axenic culture and then transferred into 500 mL of MB (Difco 0113-17-5) for growth at 20−23 °C in the dark. Cultures were subcultured every 4−6 weeks. The fungi obtained as putative endophytes were routinely maintained on MB, YMB, yeast malt agar, potato dextrose agar, and Sabouraud's dextrose agar (SDA).

Isolation and Analysis of Total Genomic DNA. The fungi grown for identification were cultured on SDA until the mycelia were approximately 3 cm in diameter. The cultures were then submitted to MidiLabs (Newark, DE) for sequence identification based on the MicroSeq D2 fungal sequencing protocol.[23] Briefly, up to 100 mg of fungal tissue were scraped from the agar surface and transferred to a tube for extraction of DNA using the MicroSeq extraction solution. The tubes were placed in a boiling water bath for 10 min. The biomass was removed by centrifugation, and the DNA was precipitated with an excess of isopropanol. The DNA was collected by centrifugation, treated with proteinase K and RNase A, and subjected to PCR analysis using primers directed to the D2 region of the large subunit (LSU) (28S) rDNA. The primers used for PCR amplify the genomic region of positions 3334 to 3630 in the *Schizosaccharomyces japonicus* LSU rDNA. PCR products were sequenced using the AmpliTaq FS DNA polymerase and dRhodamine dye terminators followed by electrophoretic sequencing on an ABI Prism 377 DNA sequencer. The sequences were matched against the GenBank and MicroSeq databases.

Chromatographic Separation and HPLC Conditions. Fungal isolates were cultured in either MB or YMB for determination of production of **1**. The biomass was separated from the medium by filtration or centrifugation depending on the nature of the culture (compact mycelium vs diffuse hyphae). The collected biomass was dried for 24 h at 70−80 °C. The dried biomass was weighed and stored at 4 °C until further processing. The dried biomass was briefly crushed with a metal spatula, loaded into a Soxhlet thimble, and extracted by Soxhlet for 6−8 h with 95% EtOH. The extract was analyzed by HPLC for **1**.

The fungal culture medium was filtered through a 0.2 μm filter to remove any cellular debris. The medium was then passed through a conditioned C_{18} solid-phase extraction (SPE) column (BakerBond, 500 mg sorbent, 6 mL volume, wide mouth) and washed with 5 mL of hexane, followed by 5 mL of 30% EtOH, and **1** eluted with 5 mL of 50% EtOH. Each SPE column was conditioned with deionized water, EtOH, and deionized water, in sequence, prior to loading of the culture medium. The SPE protocol was developed using authentic **1** (Sigma) dissolved in 500 mL of water, and the protocol was confirmed with **1** in water and culture medium (Table 1). Quantitation of samples was performed based on standard curves of five concentrations (10, 30, 50, 100, 200 μg/mL) of **1** run at the time of the samples.

Soxhlet and SPE extracts were analyzed for **1** using Zorbax ODS, Zorbax SB-C_{18}, Hypersil BDS (Alltech), and Alltima C_{18} (Alltech) columns (all columns: 5 μm, 4.6 × 250 mm) on both Hewlett-Packard

1090M and Dionex P580 systems each outfitted with PDA detectors. Samples of each extract (20 μL) were analyzed using a 65:35 to 35:65 0.05% TFA/MeOH linear gradient over 55 min (1 mL/min). Spectra were monitored at 220, 254, 275, and 300 nm. Spectrophotometric data were collected at 190−400 nm.

Brine Shrimp Lethality Assay. The brine shrimp lethality assay followed the procedures of Meyer et al.[24] The brine shrimp eggs were hatched in artificial seawater with aeration and maintained on yeast extract. When nauplii were between 3 and 7 days post-hatching, 10 nauplii were transferred by pipet into each well of a 6-well dish along with 5 mL of artificial seawater and with a volume of extract for a desired concentration. After 24 h, the total number of living and dead nauplii were counted in each well. LD_{50} was determined on the basis of a probit analysis[25] of the response compared to authentic **1**.

Acknowledgment. We are grateful to L. Kilmer, GlaxoSmithKline, King of Prussia, PA, for the MS analysis of positive extracts. We also thank the Elsa U. Pardee Foundation for Cancer Research, Midland, MI, for partial support of this research. A.L.E. and R.D. thank the Cell Biology & Biotechnology Program, Department of Biological Sciences and the College of Graduate Studies, University of the Sciences in Philadelphia, Philadelphia, PA, for support of the research leading to this paper. J.R.P. also thanks D. Newman, NCI (Frederick, MD), for useful discussions related to the implications of the research.

Supporting Information Available: Chromatographic and spectrophotometric data for the podophyllotoxin (**1**) obtained from the endophytes, DNA sequences, morphological description of the isolated endophyte fungi, and a neighbor-joining tree of the fungi. This information is available free of charge over the Internet at http://pubs.acs.org.

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Supporting Information

Two Endophyte Fungal Isolates from *Podophyllum peltatum* Produce Podophyllotoxin

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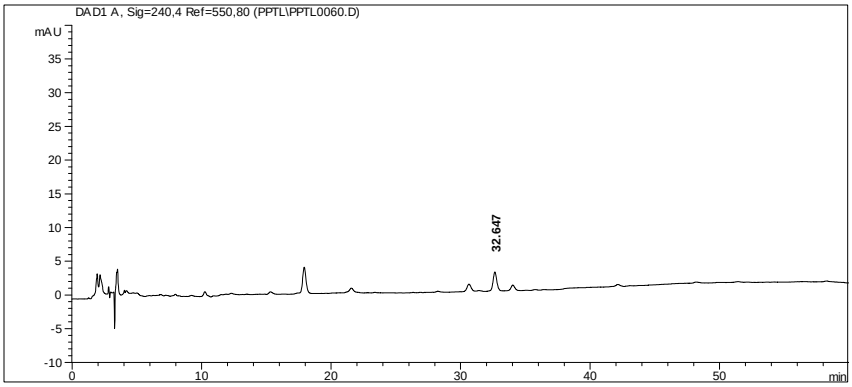

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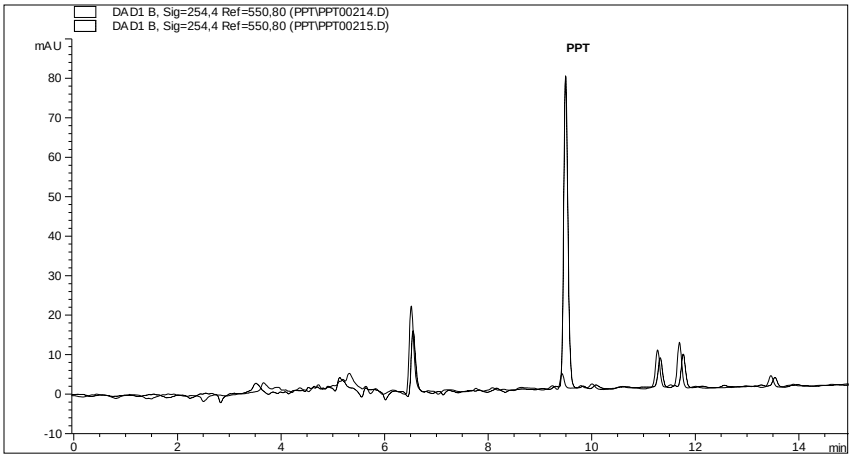

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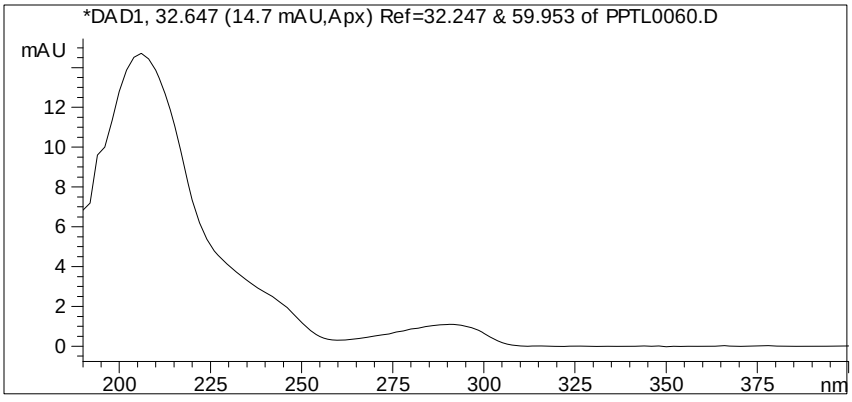

C

Figure S1. HPLC chromatograms and absorption spectrum of the fungal podophyllotoxin (1). A. HPLC chromatogram of fungal extract from PPE7 medium showing the peak at 32.647 min. **B.** HPLC chromatogram of PPE7 fungal extract in a short, screening run with authentic, plant-derived podophyllotoxin (**1)** chromatogram overlaid. **C.** Absorption spectrum of fungal podophyllotoxin (**1**) from peak shown in **A.**

PPE5
TGATCGAARGATGAAAAGCACTTKGGAAAGAGAGTTAAACAGTACGTGA
AATTGTTGAAAGGGAAGCGCTTGCAAACCAGACTTGCGGGCGGTCGATCA
TCCGAGGTTCTCCCCGGTGCACTCGATCGTTCTCAGGCCAGCATCGGTTTC
CGGGGTGGGATAAAGGCGGTGGGAATGTGGCTCTTCGGAGTGTTATAGCC
CACCGTGCAATGCCGCCACTGGGGACCGAGGACCGCGCTTCGGCTAGGAT
GCTGGCGTAATGGTTGTAAGCGACCCGTCTTGAAACACGGACCAAG

PPE 7
GGRKGATSGAARGWTGAAAAGCACTTKGGAAAGAGAGKTAAACAGTACG
TGNAAATTGTTGAAAGGGAAGCGCTTGCAACCAGACTTGCAGGCGGTCGA
TCATCCGAGGTTCTCCCCGGTGCACTCGATCGTCTTCAGGCCAGCATCGGT
TTCGGTGGCGGGATAAAGGCTCTAGGAATGTGGCTCTTCGGAGTGTTATA
GCCTAGGGTGCAATGCCGCCTACCGGGACCGAGGACCGCGCTTCGGCTAG
GATGCTGGCGTAATGGTTGTAAGCGGCCCGTCTTGAAACACGGACCAAG

Figure S2. DNA sequences obtained from each of the endophyte fungi strains that produce podophyllotoxin (1) in culture. The sequences were obtained using the methods described in the **Experimental Section**. Non-Watson-Crick base coding follows the IUPAC convention for nucleotides.

Morphological descriptions (on MB)

PPE5 – The fungus produces copious amounts of aerial, surficial and submerged hyphae. The mycelia are all dark brown to black with dark tan, cottony centers. The aerial hyphae are almost all medium to dark tan, slender, with pointed tips. The surface hyphae are hyaline to dark brown or black, slender with pointed growing tips. The is no obvious presence of toroid cells. There has been no evidence of sporulation after six years in culture on a variety of media and growing conditions (light, dark, 20 °C, 4 °C for up to one year). Microscopically, the hyphae are highly and irregularly branched. There are three classes of hyphae, all septate: 1) highly branched with long cells 4-6 µm wide × 25-30 µm long, with slender hyaline tips; 2) shorter, broader cells somewhat swollen in the center, 6-8 µm wide × 16-18 µm long, mostly dark brown; 3) shorter cells with distinct joints (arthropod), 4-5 µm wide × 8-10 µm long.

PPE7 – The fungus produces an arborescent growth with aerial, surface and submerged hyphae. The principal mycelium color is dark olive green to black with hyaline growing tips. The aerial and agar surface hyphae are slender with pointed tips. The submerged hyphase consist of strings of toroid cells. The strictly aerial hyphae are a mixture of black and medium tan. Microscopically, the aerial hyphae are dark brown or black, septate, 1.5-3.5 µm wide × 20-35 µm long. Submerged hyphae with subtoroid cells 3.5 × 8 µm. Conidia (rare) hyaline, 1.5-3.5 µm in diameter.

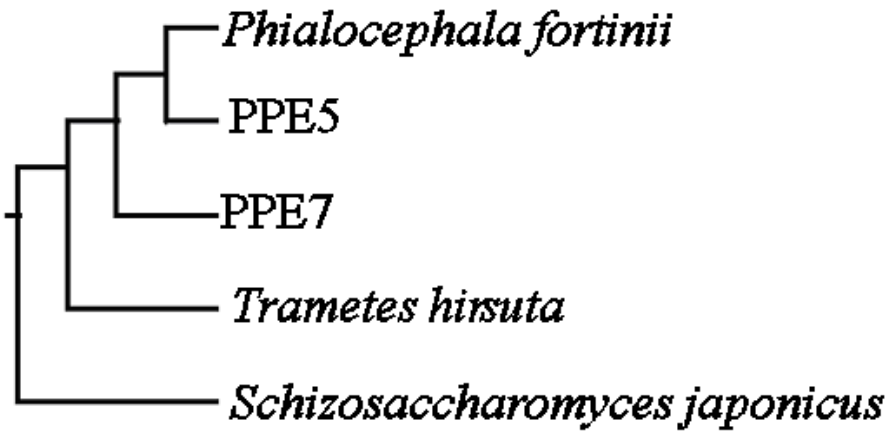

Figure S3**. Neighbor-joining tree of the consensus sequence of *Phialocephala fortinii*, PPE5, PPE7, and *Trametes hirsuta*[7].** The tree was constructed based on multiple alignments using ClustalW (http://align.genome.jp). The tree is rooted by comparison to *Schizosaccharomyces japonicus*. All alignments were done using a comparable region of the 28S (LSU) rRNA gene.