# An endophytic *Coniochaeta velutina* producing broad spectrum antimycotics

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**An endophyte (PC27-5) was isolated from stem tissue of Western hemlock (*Tsuga heterophylla*) in a Pacific Northwest temperate rainforest. Phylogenetic analyses, based on ITS-5.8S rDNA and 18S rDNA sequence data, combined with cultural and morphological analysis showed that endophyte PC27-5 exhibited all characteristics of a fungus identical to *Coniochaeta velutina*. Furthermore, wide spectrum antimycotics were produced by this endophyte that were active against such plant pathogens as *Sclerotinia sclerotiorum*, *Pythium ultimum*, and *Verticillium dahliae* and lethal to *Phythophthora cinnamomi*, *Pythium ultimum*, and *Phytophthora palmivora* in plate tests. The bioactive components were purified through organic solvent extraction, followed by silica column chromatography, and finally preparative HPLC. The minimum inhibitory concentration of the active fraction to *Pythium ultimum*, which was gained from preparative HPLC, was 11 μg/ml. UPLC-HRMS analysis showed there were two similar components in the antimycotic fraction. Their molecular formulae were established as $C_{30}H_{22}O_{11}$ (compound I) and $C_{30}H_{22}O_{10}$ (compound II) respectively, and preliminary spectral results indicate that they are anthroquinone glycosides. Other non – biologically active compounds were identified in culture fluids of this fungus by spectral means as emodin and chrysophanol - anthroquinone derivatives. This is the first report that *Coniochaeta velutina* as an endophyte produces bioactive antifungal components.**

**Keywords:** antimycotics, endophyte, *Coniochaeta velutina*, bioassays, SEM

## Introduction

Endophytes are described as microorganisms that colonize the tissues of healthy plants without causing apparent pathological symptoms in them (Carroll, 1988). Usually, the roles of endophytes might range from symbiotic to borderline pathogenic (Bacon and White, 2000; Strobel and Daisy, 2003). It appears that only a limited number of fungi are capable of establishing an endophytic relationship with plants, perhaps because they can be inhibited or killed by toxic substances within the plant tissues. Endophytes may protect plants by producing a series of secondary metabolites, which also may have potential in human medicine (Strobel and Daisy, 2003; Yan *et al.*, 2014). And, it also seems that the relationship between endophytes and host plants is dynamic and depends on the environmental conditions of the host plant, the microorganisms involved and the ecosystem in general. Further research is necessary to reveal completely all of the details of any plant-endophyte relationship.

The greatest diversity of endophytic species occurs in areas that are relatively undisturbed and possess high plant diversity (Strobel, 2003; Strobel and Daisy, 2003). Significant biological diversity also means comparatively high appearance of chemical diversity (Strobel *et al.*, 2005). Sampling from any undisturbed bio-diversified area of the world offers the prospect for discovery of novel microbes, unique biologically active products and good models to explore the relationship between endophytes and host plants (Strobel and Daisy, 2003).

The Olympic Peninsula is the largest temperate rainforest in the United States and thus attracted our research interest since it possesses high plant diversity and has areas that are relatively undisturbed. Such an ecosystem hosts an abundance of endophytes with their corresponding bioactive substances (Alaback, 1991). One valuable, biologically-active fungus, identified as *Coniochaeta velutina* (PC27-5), was isolated from a healthy Western hemlock (*Tsuga heterophylla*) which was collected in this area. Most isolates of the genus *Coniochaeta* have been reported as opportunistic pathogens isolated from dung, necrotic wood, soil, and plant surfaces (Damm *et al.*, 2010; Chang and Wang, 2011). Recently, a species of *Coniochaeta* with some antimicrobial properties was isolated from a rainforest in Thailand (Kokaew *et al.*, 2011). *C. velutina* PC27-5 existed in the stem tissue of Western hemlock as an endophyte. *C. velutina* PC27-5 can inhibit, and in some cases kill, some plant pathogenic fungi. This report details the isolation and characterization of this endophyte and presents information on the antifungal agents and other related compounds that it produces.

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## Materials and Methods

### Fungal isolation and storage

In the summer of 2012, several small stems were removed from healthy specimens of Western hemlock (*Tsuga heterophylla*) and transported back to Montana State University from the Pacific Coast. The isolation procedures to acquire the endophytes from the plant materials followed those previously described (Strobel *et al.*, 2000). In brief, the twig pieces were soaked in a 7/3 ethanol/water (v/v) solution for surface disinfection, quickly flame-sterilized, and then the outer bark was removed with a sterile scalpel. Small pieces of inner bark were aseptically transferred to the surface of water agar (WA) and glycerol-arginine medium (GAM) (Strobel *et al.*, 2000; Tomsheck *et al.*, 2010). After several days of incubation at 25°C, hyphal tips of developing fungi were aseptically removed and placed on potato dextrose agar (PDA), thus establishing pure fungal cultures. About ten days later, the fungi were transferred to another PDA plate to assess their antibiotic activity against test plant pathogens. The isolated endophytes were preserved by growing a pure fungal culture on sterile barley seeds and storing the seeds at -70°C (Tomsheck *et al.*, 2010). The fungus PC27-5 is deposited as number 2406 in the living mycological collection of Montana State University.

### Morphology and Scanning Electron Microscopy (SEM)

The cultural morphological features of endophyte PC27-5 were explored by observing the fungus on several media for one to five weeks, including PDA, oatmeal agar (OA), nutrient agar (NA), and PDA with the stem fragments of the host plant. In order to acquire detailed information on the morphological features of the fungus, scanning electron microscopy (SEM) was performed, according to the protocol outlined by Ezra *et al.* (2004). The observed fungal material was about eight weeks old, having grown on PDA with the stem material of host plant. The samples were slowly dehydrated in ethanol and then critically point dried, coated with gold and examined with an FEI XL30 SEM FEG with high vacuum mode using an Everhart-Thornley detector (Tomsheck *et al.*, 2010).

### Analysis of 18S rDNA and ITS-5.8S rDNA

The 18S rDNA sequence is helpful in identifying a fungus at or above the genus level, whereas the internal transcribed spacer (ITS) sequence is useful in discriminating at or near the species level (Worapong *et al.*, 2001). Genomic DNA was extracted from fungus grown on PDA medium for two weeks (Strobel *et al.*, 2010). DNA templates were prepared with the Prepman Ultra Sample Preparation Reagent (Applied Biosystems), according to the manufacturer's guidelines. The phylogenetic analysis of endophyte PC27-5 was carried out by acquisition of 18S rDNA and ITS-5.8S ribosomal gene sequence using the polymerase chain reaction (PCR). The ITS-5.8S rDNA regions of endophyte PC27-5 were amplified with universal ITS primers ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGT AACAAGG-3′) (White *et al.*, 1990). The PCR conditions were set as follows: initial denaturing at 94°C for 4 min,

followed by 30 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. The 18S rDNA sequences were amplified using two pairs of universal primers (White *et al.*, 1990), which were: NS1 (5′-GTAGTCATATGCTTG TCTC-3′) as forward, NS4 (5′-CTTCCGTCAATTCCTTT AAG-3′) as reverse; NS3 (5′-GCAAGTCTGGTGCCAGC AGCC-3′) as forward, NS8 (5′-TCCGCAGGTTCACCTA CGGA-3′) as reverse. The PCR conditions were as follows: initial denaturing at 94°C for 4 min, followed by 30 cycles of denaturing at 94°C for 50 sec, annealing at 55°C for 50 sec, extension at 72°C for 70 sec, and a final extension at 72°C for 10 min. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN), according to the manufacturer's instructions, and sequenced by the University of California-Berkeley.

Raw sequences were edited using FinchTV (Dymond *et al.*, 2009). The amplified sequence of endophyte PC27-5 and published sequences retrieved from GenBank (www.ncbi. nlm.nih.gov), which were selected according to the BLAST results on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and cultural and morphological features of endophyte PC27-5 on different media, were aligned with ClustalX2. Further phylogenetic analyses were performed by MEGA4 using Neighbor-Joining (NJ) method with kimura-2-parameter substitution model (Tomsheck *et al.*, 2010). The resulting trees were evaluated with 1,000 bootstrap replications.

### Bioassay tests of endophyte PC27-5

The following plant pathogens were used in the bioassay tests of endophyte PC27-5: *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum*, *Pythium ultimum*, *Botrytis cinerea*, *Verticillium dahliae*, *Cercospora beticola*, *Colletotrichum lagenarium*, *Ceratocystis ulmi*, *Trichoderma viridae*, *Fusarium solani*, *Geotrichum candidum*, *Mycosphaerella fijiensis*, and *Phytophthora palmivora*. All microorganisms used in the bioassay test were obtained from the Department of Plant Sciences, Montana State University. All test microorganisms were cultivated on PDA at 25°C and only freshly transferred cultures were used in the bioassays.

A 21-day-old culture of endophyte PC27-5 was tested against all of the listed microorganisms. Each target test microbe (ca. 3 mm$^3$ agar plus) was placed on a PDA Petri plate at a 10-mm distance from the edge of a PC27-5 colony. Simultaneously, test microbes were inoculated onto fresh PDA as controls. The filamentous fungi were quantitatively assessed based on multiple measurements of growth extending from the edge of the growing culture and compared to the growth of corresponding controls (Tomsheck *et al.*, 2010). Inhibition percentages were calculated at 4 days for most of the test microbes, except for *Fusarium solani* and *Geotrichum candidum*, which were calculated at 2 days. The time at which the optimum production of the antimycotics occurred was determined by inoculating test organisms on PDA with cultures of endophyte PC27-5 that were from one to five weeks old.

### Assay and purification of the bioactive compounds

*Pythium ultimum*, the most sensitive pathogen, was employed

in a series of steps for bioassay-guided fractionation in order to acquire one or more fractions with antifungal activity. Various fractions with bioactivity were subjected to minimum inhibitory concentration (MIC) tests in order to assess the relative activities of each preparation. To this end, 10 μl of a methanol test sample solution were placed into each well of a 96 well plate. The test solutions held different concentrations of the sample, and the control was 10 μl methanol. After the solvent methanol evaporated totally, 100 μl potato dextrose broth (PDB) were placed into each well. Agar plugs (1 mm$^3$) with fresh *P. ultimum* were inoculated into each well. Observations of the plates were made after 24 h of incubation at 25°C in order to acquire the MIC values with no growth in the well taken as that value.

The fermentation broth of endophyte PC27-5 was obtained under shaking conditions at 150 r/m and was harvested 3 weeks after being incubated in PDB at 25°C. The active component was purified by organic solvent extraction, silica column chromatography, and preparative high performance liquid chromatography (RP-HPLC) (Calhoun, 1992; Stierle *et al.*, 1993; Wang *et al.*, 2010). A 3-week-old, 4 L fermentation broth of endophyte PC27-5 was centrifuged for 10 min at 9000 r/m, and then the supernatant was extracted with dichloromethane 1/1 (v/v). The solvent was removed by rotary evaporation at 50°C, yielding the organic solvent extract. The organic solvent extract was dissolved in 20 ml dichloromethane and 4 ml were applied to a 40 × 450 mm column containing 100 g of silica gel. It was washed with 450 ml of dichloromethane, and then eluted successively with dichloromethane/methanol, with the content of methanol being increased from 10% to 70% in succeeding 10 percent increments. The elution volume of each concentration was 450 ml. Finally, 450 ml of methanol were applied. Assays were done on each fraction, and the active fraction was further isolated by preparative HPLC.

Preparative HPLC was performed on an Agilent 1260 liquid chromatography system equipped with an Agilent Zorbax SB-C18 column (9.4 × 150 mm, 5 μm). The column was maintained at 30°C with a flow rate of 10 ml/min, and was eluted by water/acetonitrile with a gradient system from 80/20 to 20/80 (v/v) in 20 min. Samples were collected and bioactivity determinations were made.

In another case, the organic solvent extract was dissolved in methanol and applied to an RP-C18 chromatography column (24 × 240 mm). It was washed with 100 ml MeOH-H$_2$O 2/8 (v/v), then eluted with 100 ml MeOH-H$_2$O 4.5/5.5 (v/v), 100 ml MeOH-H$_2$O 8/2 (v/v), and 100 ml 100% methanol. The main fractions were collected, applied to the silica gel column (20 × 240 mm), and eluted with about 250 ml petroleum ether/acetone 9/1 (v/v). The silica gel column yielded two main pigmented components, which were collected and designated as compounds III and IV.

Various column fractions were analyzed on an Agilent 1100 liquid chromatography system equipped with an Agilent Zorbax SB-C18 column (4.6 × 150 mm, 5 μm). The column was maintained at 30°C with a flow rate of 1 ml/min, which was eluted by water/acetonitrile with a gradient system from 70/30 to 0/100 (v/v) in 10 min.

Determination of molecular weights of purified compounds was performed using a Shimadzu LC-MS-IF-TOF system equipped with a Thermo C18 column (2.1 × 100 mm, 1.9 μm). The column was maintained at 30°C with a flow rate of 200 μl/min, which was eluted by water/acetonitrile (add 0.05% v/v formic acid) in a gradient system from 95/5 to 5/95 in 12 min. A diode array detector was used in-line following the column and prior to the mass spectrometer.

The mass spectrometer used was a Shimadzu LC-MS-IT-TOF with-ESI source: resolution is approximately 10,000 FWHM. Source parameters were as follows: drying gas 100 kpa, nebulizing gas flow, 1.5 L/min, spray voltage, 4.50 kV or - 3.50 kV. Spectra were collected in positive and negative modes from 100 to 2000 m/z. MS and MSn spectra were obtained in the automatic mode.

NMR spectra were obtained using an AV-600 MHz spectrometer in methanol-$d_4$ with TMS as an internal standard.

## Results and Discussion

### Fungal isolation and morphological features

**Fungal isolation and colony characters:** Endophyte PC27-5 was isolated from surface disinfected stem tissue of Western hemlock, which was collected from the Olympic Peninsula. Preliminary bioassay results showed endophyte PC27-5 was inhibitory to *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum*, and *Pythium ultimum*, which encouraged further work on this organism, including its identification.

Overall, the colonies of endophyte PC27-5 on most media were moderately growing, and dark brown, dark green, bright yellow, and ivory-white. On PDA: the colony diameter was 34–38 mm with felt-like aerial mycelium in 2 weeks at 25°C. The colony surface was dry, wrinkled, and dark brown in the center and white to amber on the margins (Fig. 1A). Sometimes, seta formed on PDA after two or three weeks (Fig.
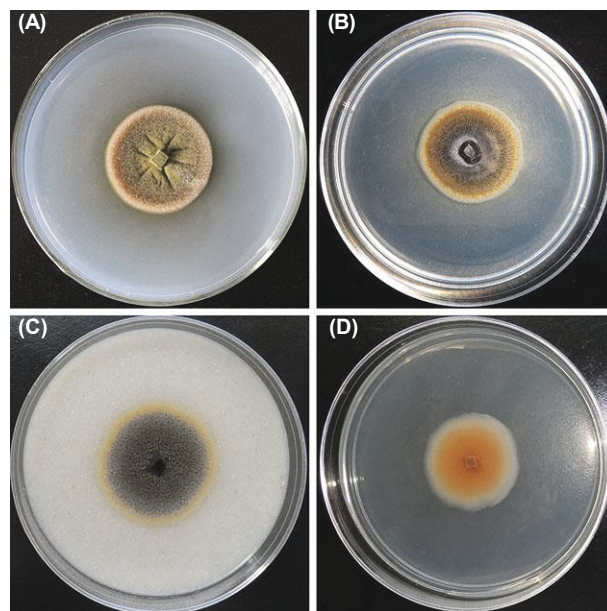


**Fig. 1. Colony morphological features of endophyte PC27-5 on different media.** [(A, B) On PDA for 2 weeks, note seta formed on the surface of PDA, (C) On OA for 2 weeks, (D) On NA for 2 weeks.]
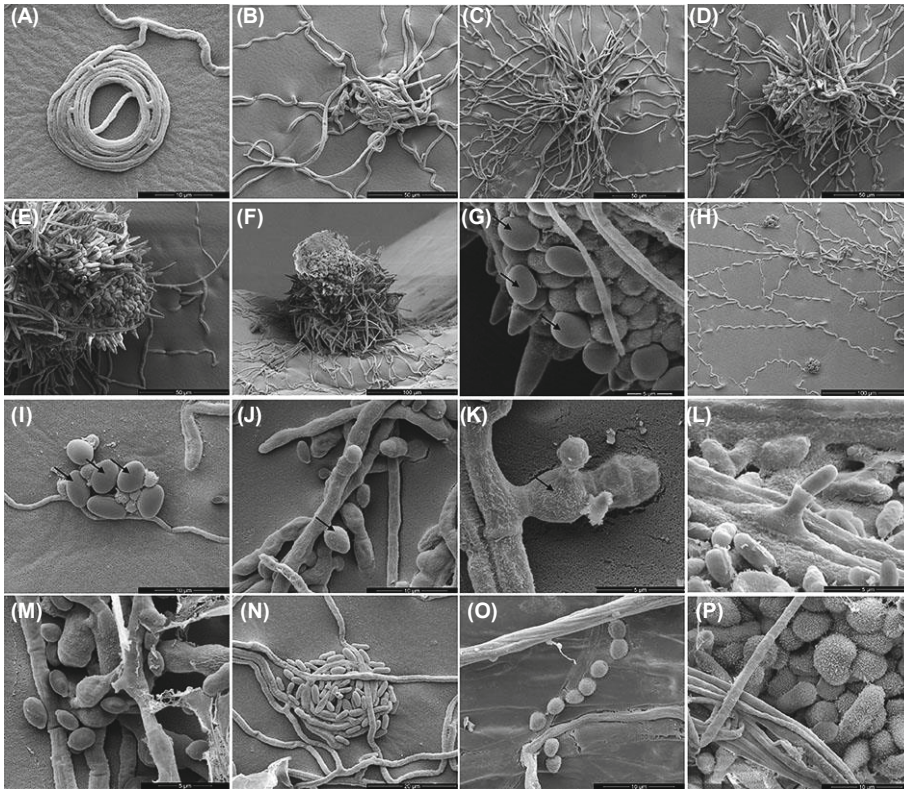
**Fig. 2.** **Scanning electron micrographs of endophyte PC27-5 gown on PDA with the stem fragments of host plant.** [(A) Mycelium characteristic curled into a circle, (B-F) Intertwining of hyphae with the gradual formation of a fruiting body, (G) Ascospores on the top of a perithecium, (H-I) Hyphae and ascospores spread on the surface of the medium, (J-K) Conidiogenous cell, (L-M) Conidiogenous cell, conidia and cup-shape collarette, (N) Conidia spread in a mass, (O-P) Other different shapes spores.]

1B). On OA: the colony diameter was 35–39 mm with fluffy, aerial mycelium in 2 weeks at 25°C, with dark brown coloration in the colony center and bright yellow margins (Fig. 1C). On NA: the colony was soft, moist, without aerial hyphae and orange in the center, with creamy margins (Fig. 1D).

**Scanning Electron Microscopy results:** Eight-week-old samples of endophyte PC27-5 on PDA with the stem material of host plant were viewed by SEM. The perithecia that formed were lageniform, solitary, and superficial on the stem material of Western hemlock and on the surface of PDA. The hyphae grew into a circular shape on the surface of the medium (Fig. 2A). The mycelia gradually intertwined, matured, and formed dark brown perithecia (Fig. 2B–F). The outer wall of the perithecium consisted of dark brown *textura angularis*, setose, with a central ostiole, up to 143 µm diameter (Fig. 2F). Ascospores were hyaline, smooth-walled, and 3–4.5 × 4.5–7 µm (Fig. 2G–I). The setae were brown, cylindrical, tapering to a round tip, generally straight, aseptate, smooth-walled or verruculose, 2.0–4.5 µm wide, and up to 25 µm long (Figs. 1B and 2D–F). Vegetative hyphae were hyaline and 1.5–2.5 µm wide (Fig. 2H–K). Conidiophores were reduced to Conidiogenous cells (Fig. 2J–K). Conidiogenous cells were enteroblastic, hyaline, with discrete phialides that were cylindrical to ventricose, with 1–3 µm long necks (including collarette) and collarette openings 1–2 µm wide (Fig. 2J–M). Sporulation was abundant (Fig. 2L–N). Conidia were hyaline, single celled, smooth-walled, cylindrical with round ends or with one end slightly acute, and 1.0–2.0 × 2.0–6.5 µm (Fig. 2L–N). Some verruculose, irregular spores were formed on the surface of the PDA (Fig. 2O–P).

**Phylogenetics analysis of 18S rDNA and ITS-5.8S rDNA**

18S rDNA sequences of endophyte PC27-5, which were amplified by primer pairs NS1-NS4 and NS3-NS8, possessed 1074 bases and 1179 bases, respectively. The whole length 18S rDNA sequence was assembled to be 1710 bases by CAP3 Sequence Assembly Program. 18S rDNA of endophyte PC27-5 was submitted to GenBank with assigned deposit number KP776994. 506 bases of ITS-5.8S rDNA sequence of endophyte PC27-5 were amplified by universal primers ITS4-ITS5. ITS-5.8S rDNA of endophyte PC27-5 was submitted to GenBank with assigned deposit number KP776995. BLAST results of 18S rDNA and ITS-5.8S rDNA on NCBI indicated endophyte PC27-5 belonged to *Leotiomycetes* class of *Pezizomycotina* subphylum. Based on the BLAST results on NCBI and morphological features of endophyte PC27-5, the similar strains of *Collophora* sp., *Teberdinia hygrophila*, *Torrendiella eucalypti*, *Oidiodendron* sp., *Coniochaeta* sp., and *Physcia stellaris* were used in phylogenetic analysis. *Geotrichum* sp. was used as an outgroup for the phylogenetic analysis of endophyte PC27-5. The main clades in the phylogenetic tree represented different classes within Ascomycota subphylum Pezizomycotina. Phylogenetic analytical results were based on the sequence of 18S rDNA, which indicated that endophyte PC27-5 belongs to the genus *Coniochaeta* (Fig. 3A). Further analytical results, based on ITS-5.8S rDNA, indicated endophyte PC27-5 was close to the species designation of *C. velutina* and within the same minimum clade (Fig. 3B). The cultural and morphological data were strongly supportive of the molecular data, allowing the identification of PC27-5 as *C. velutina* (Davey *et al.*, 2010; Chang and Wang, 2011).
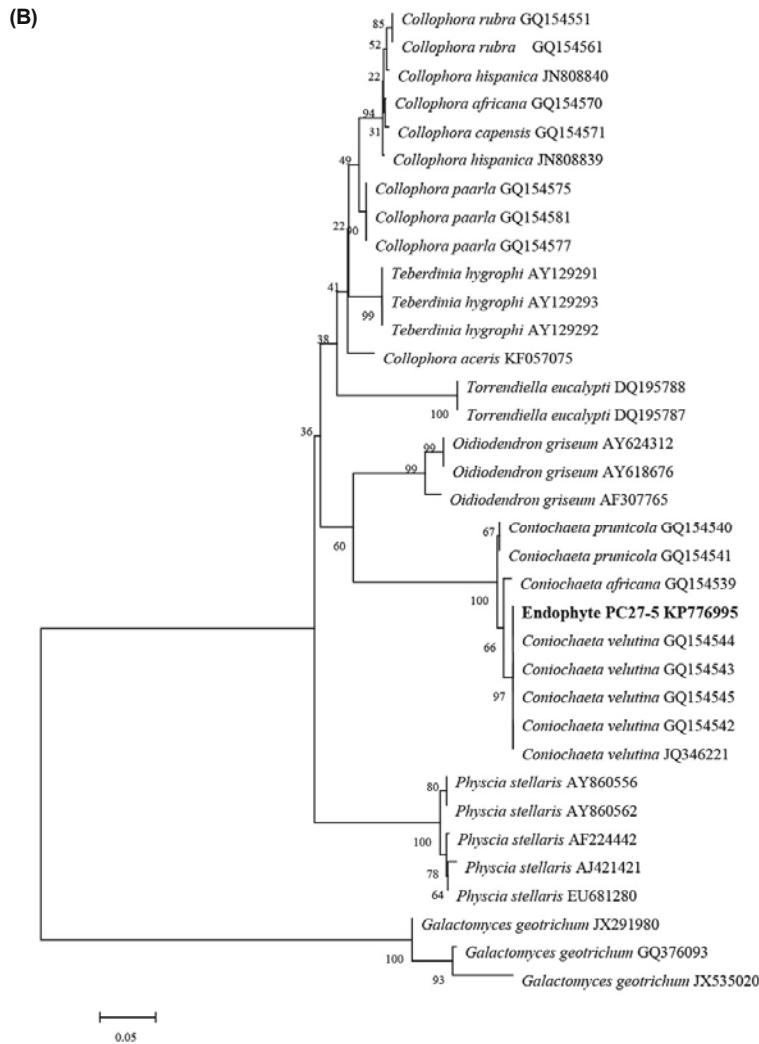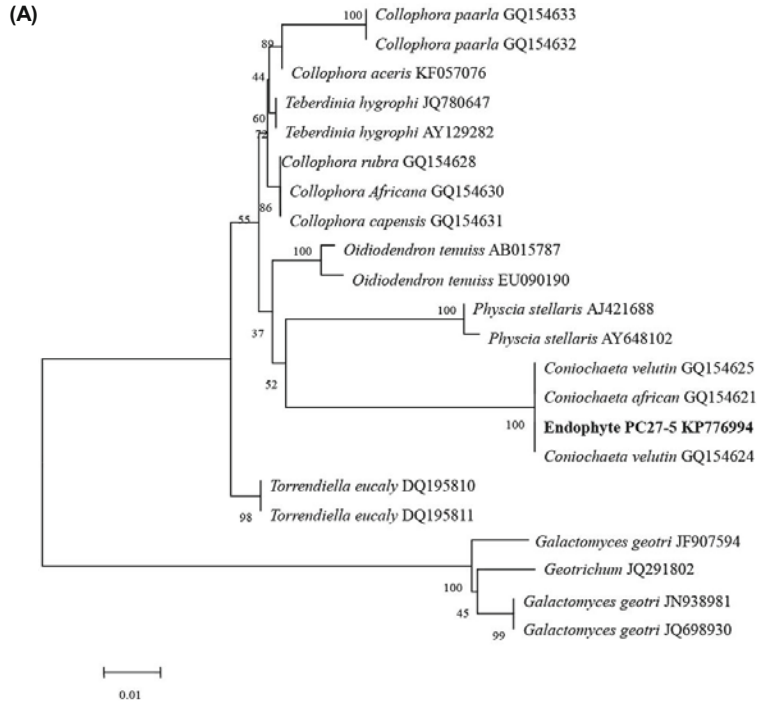
**(A)**



**(B)**



**Fig. 3.** (A) **Phylogenetic tree of endophyte PC27-5 based on 18S rDNA.** The tree was constructed by MEGA4 using Neighbor-Joining (NJ) method with kimura-2-parameter substitution model. The resulting tree was evaluated with 1000 bootstrap replications. (B) **Phylogenetic tree of endophyte PC27-5 based on ITS-5.8S rDNA.** The tree was constructed by MEGA4 using Neighbor-Joining (NJ) method with kimura-2-parameter substitution model. The resulting tree was evaluated with 1000 bootstrap replications.
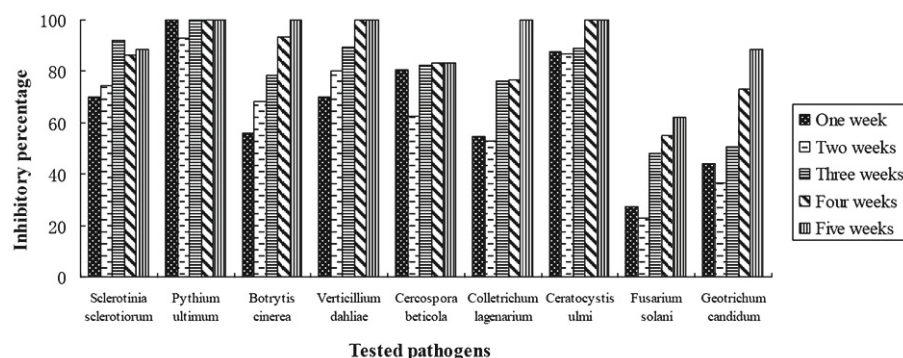
Fig. 4. **Inhibitory activity of cultures of *Coniochaeta velutina* PC27-5 at different ages to 9 plant associated fungi.** Inhibitory values were calculated as a percentage of growth inhibition as compared to control test organisms 4 days after inoculation (most test pathogens) or for 2 days with *Fusarium solani* and *Geotrichum candidu.*

## Biological activities of metabolites of *Coniochaeta velutina* PC27-5

The expression of antibiotic activity of *C. velutina* PC27-5 against the various test microorganisms was dependent on the age of the culture. A progressive time course assay using nine different pathogens as targets was designed to determine the time point that was suitable for the production of antibiotics of *C. velutina* PC27-5. The cultivation time ranged from one to five weeks. The results indicated that the ideal maximum inhibitory activity can be acquired at five weeks; the inhibitory activity did not change much from three weeks to five weeks for most pathogens (Fig. 4), which implies that three weeks is the optimal time for harvesting the antifungal compounds. The most sensitive pathogen, *P. ultimum,* was totally inhibited by *C. velutina* PC27-5 from 3 weeks to 5 weeks (Fig. 4).

An expanded bioassay test involving more microorganisms revealed that the inhibitory activity of the 3-week-old *C. velutina* PC27-5 culture varied substantially against different test microorganisms (Table 1). *Trichoderma viridae* was not inhibited at all. *Phythophthora cinnamomi, Phytophthora palmivora*, and *Pythium ultimum* were completely inhibited and killed, which was determined since they did not grow after being transferred to fresh PDA plates.

**Table 1.** Effects of 3-week-old culture of *C. velutina* PC27-5 on various fungi

| Test organism | Percent inhibition | | D or A |
|---|---|---|---|
| *Phythophthora cinnamomi* | 100.00 | 0.00 | D |
| *Sclerotinia sclerotiorum* | 92.03 | ±4.06 | A |
| *Pythium ultimum* | 100.00 | 0.00 | D |
| *Botrytis cinerea* | 78.53 | ±8.06 | A |
| *Verticillium dahliae* | 89.60 | ±11.61 | A |
| *Cercospora beticola* | 82.43 | ±14.60 | A |
| *Colletrichum lagenarium* | 76.10 | ±17.48 | A |
| *Ceratocystis ulmi* | 88.87 | ±12.53 | A |
| *Trichoderma viridae* | 0.00 | 0.00 | A |
| *Fusarium solani*[a] | 48.13 | ±8.86 | A |
| *Geotrichum candidum*[a] | 50.77 | ±27.45 | A |
| *Mycosphaerella fijiensis* | 50.00 | ±14.20 | A |
| *Phytophthora palmivora* | 100.00 | 0.00 | D |

Inhibition values were calculated as a percentage of growth inhibition as compared to control test organism in inoculated 4 day exposure (a- 2 days). Tests were conducted in triplicate and results varied as indicated by standard deviation. All organisms were viable.
D, The pathogen cannot grow again after transferring it from test plate to fresh PDA
A, The pathogen can grow after transferring it from test plate to fresh PDA

## Purification of various cultural components of *Coniochaeta velutina* PC27-5

The crude sample (4 L) extracted with dichloromethane yielded an MIC of 300 μg/ml with *P. ultimum.* The bio- fractions eluting from the silica gel column at dichloromethane-methanol concentrations of 70/30, 60/40, and 50/50 (v/v) shared a similar chromatogram map upon HPLC analysis, and when mixed together, possessed an MIC of 200 μg/ml to *P. ultimum.* This mixture was applied to preparative RP-HPLC for further purification. The bio-active component (1.1 mg) was obtained from the preparative RP-HPLC at RT 13.3 min. The bioassay results showed that this bio-active fraction was highly inhibitory to *P. ultimum* and *S. sclerotiorum*, with an MIC at 11 μg/ml to *P. ultimum.*

The $H^1$ NMR of this bioactive fraction revealed the presence of one or more benzene rings (6.8 and 7.4 ppm), the potential presence of a β-glycosidic moiety (3.2–3.7 ppm and 4.5 ppm) and up field protons consistent with $CH_2$, $CH_3$ functionalities in the structure (1.3–2.3) (Van der Veen, 1963; Silverstein *et al.*, 1991; Agrawal, 1992). The UPLC-ESI-MS analysis of this bio-active fraction, which was a yellowish powder, indicated it had two UV absorption peaks recorded at 263 nm on the UPLC column, which were at RT 8.50 min (Compound I) and 10.05 min (compound II), respectively. Compound I possessed a molecular formula of $C_{30}H_{22}O_{11}$, as evidenced by HRESIMS at m/z a weight 557.1051 $[M+H]^+$. The molecular formula of compound II was established as $C_{30}H_{22}O_{10}$ by HRESIMS ($[M+H]^+$ at m/z 541.1106). The millimolar extinctions for the UV absorbances of compound I were at 239 Є = 12.51, peak 254 Є = 10.19, and peak 282 Є = 5.56. The millimolar extinctions for the UV absorbances for compound II were at peak 248 Є = 108, peak 277 Є = 72, peak 282 Є = 63, and peak 392 Є = 126. In each case, the UV absorbances and the extinctions suggest the presence of a highly conjugated ring system with alternating single and double bonds. The data are consistent with a basic anthraquinone structure. Also, it appears that the two components are chemically related, since they have similar UV absorbances, similar molecular weights, and the same 20 degrees of unsaturation. Based on an intensive literature search and comparisons of the spectral data on hand, it appears that these bioactive compounds are unique.

One of the problems with getting final structural solutions to compounds I and II was the difficulty in separating the two components on the RP- HPLC- $C_{18}$ column. Once this
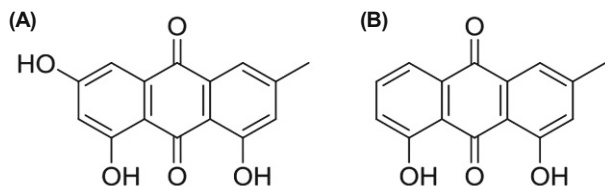
**Fig. 5.** Molecular Structures of Compound III (A) and Compound IV (B).

problem is solved, the structural solution will soon be achieved. The bioactivity of these compounds against filamentous fungi is as impressive as those reported for many commonly used synthetic fungicides.

Two other pigmented compounds were isolated as metabolites from *C. velutina* PC27-5 culture fluid. The compounds appeared in the 80%–100% MeOH-H$_2$O fraction during RP-C18 chromatography, as described above. They were further separated by silica gel chromatography, which was eluted with petroleum ether/acetone 9/1 v/v 250 ml. Compound III, which was a yellow to red powder, appeared at 140–170 ml eluted volume and Compound IV, an orange to red powder, appeared at 40–80 ml eluted volume. The HPLC analysis results (outlined above) showed that the retention times of the pigments were 7.0 min (Compound III) and 8.7 min (Compound IV). These two pigments were identified as Emodin (III) (Ghosh *et al.*, 2014) and Chrysophanol (IV) (Zhou *et al.*, 2014), respectively on the basis of NMR and MS data (Fig. 5). Bioassay tests on these compounds revealed that they had little to no activity. Nevertheless, their presence in the culture fluid may eventually provide some guidance to the structural determination of compounds I and II.

## Conclusion

The results of this work clearly demonstrate the presence of an antimycotic-producing fungus as an endophyte in the Western hemlock. This fungus was identified as *Coniochaeta velutina,* based on the molecular, cultural and morphological features. The organism grew moderately on most media and was characterized by the presences of dark brown perithecia, which formed on the surface of PDA with host plant materials. The perithecia were entirely covered by seta, but sometimes seta did form on the surface of PDA directly. The asexual stage of *C. velutina* was enteroblastic, and cylindrical conidia were released from conidiogenous cells with collarettes. The phylogenetic analysis results indicated the ITS-5.8S rDNA of endophyte PC27-5 and most strains of *C. velutina* shared over 99% identity and were within the minimal clade. Overall, most features of *C. velutina* PC27-5 were consistent with those of authentic *C. velutina* described in the literature (Davey *et al.*, 2010; Chang and Wang, 2011).

The genus *Coniochaeta* has been isolated in different environments. Usually, *Coniochaeta velutina* has been reported as an opportunistic pathogen of woody hosts. Recent studies on the biology of an isolate of *C. velutina* in mosses, however, indicated that it was not pathogenic to any of the host plants tested (Davey *et al.*, 2010). Consequently, it will be a very interesting to understand the role of *C. velutina* in its

host plant Western hemlock. This is the first report of *C. velutina* on the healthy tissue of Western hemlock as an endophyte and the first report of the antimycotic agent produced by *C. velutina*. The results of this paper indicate that *C. velutina* can establish an endophytic relationship with a host plant. Furthermore, it may be that *C. velutina* contributes to the enhancement of host resistance to pathogens through the production of antifungal agents. It also appears that these antifungal agents possess enough intrinsic biological activity to be potentially important for the control of fungal pathogens.

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