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Isolation of Endophytic Fungi from Douglas-Fir (*Pseudotsuga menziesii*) Foliage with Bioprospecting Potential for Natural Pharmaceuticals

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Abstract

Baseline inventories of fungal endophytes in Pacific Northwest forests are rare. Endophytic fungi represent a tremendous potential for production of New Chemical Entities (NCE's) for natural products and pharmaceuticals derived from bioactive metabolic compounds. This study reports on additions of a number of new species to the baseline inventory of Douglas-fir fungal endophytes. In addition, we review the literature on these reported species with a focus on those that have the potential for production of bioactive compounds. A randomized block design was used to assess endophyte populations between seed sources along a latitudinal gradient among a reciprocal provenance study of Douglas-fir. Needles were surface sterilized and underwent plate culturing. Isolated fungal colonies were identified through Sanger sequencing and analyzed through BLAST. In total, 46 unique isolates from 39 taxa were identified from 215 needles. Nearly 70% of species identified have been shown to produce bioactive compounds. Bioprospecting of fungal endophytes for new and unique pharmaceuticals and other natural products is certainly important, particularly in light of the frequency of endophytes capable of producing bioactive compounds shown in this and other studies.

Keywords: Bioprospecting; Douglas-fir; Fungal Endophytes; Natural Pharmaceuticals

Introduction

Natural products, naturally derived metabolites and/or by-products from microorganisms, plants, or animals [1], have long been known to be a leading source of molecules for drug and new product discovery [2-4] as natural selection has been found to be superior to combinatorial chemistry for discovering novel substances that have the potential to be developed into new pharmaceutical products [5]. Surveys of new chemical entity pharmaceuticals (NCE's) [2,6] reported that nearly 70% of anti-infective, 70% of antimigraine, and nearly 80% of anticancer NCE's were natural product or natural product derivatives.

Early bioprospecting was motivated with the discovery of Taxol as an endophyte derivative. This was short-lived, as fungal culture-based production was not sustainable nor competitive with plant cell extractives [7]. Early laboratory techniques and technologies certainly limited opportunities to explore, in particular, the roles of microorganisms as repositories of NCE's. Additionally, pharmaceutical companies tended to deemphasize bioprospecting in favor of combinatorial chemistry using mass-produced combinatorial libraries to develop NCE's, but this failed to produce anticipated returns [6]. Finally, the presence of orphan biosynthetic pathways that could not be replicated in laboratory situations added to the limitations of bioprospecting [8].

Since the shift away from endophyte bioprospecting, our understanding of microbial biodiversity, particularly within plant tissues (endophytes) has greatly advanced along with discoveries of novel biochemistry and secondary metabolite production. Endophytes are well suited for bioprospecting of NCE's. They are more metabolically active than free-living fungi, given the adaptive evolution of metabolic pathways necessary for within-host survival. Opportunities for bioprospecting are enhanced by the fact that unlike plants and animals, microorganisms represent the most diverse and yet least discovered entities with estimates of less than 5% of fungi and 1% of bacteria yet described [9]. Therefore, characterization of microbiome species is a necessary baseline step

to provide information for bioprospecting opportunities.

The Pacific temperate rainforest zone in North America is home to some of the most highly productive forests in the world. The zone is characterized by high rainfall and moderate temperatures year-round, which provide ideal conditions for conifer growth [10]. Several studies have attempted to describe endophytes in west coast conifers but all of these relied on traditional plate culturing [11-14] without the advantage of newer molecular procedures. Studies that characterize endophytes by molecular methods in west coast Douglas-fir are in their early stages [15]. This study was undertaken to add to the baseline data characterizing endophytes of the Pacific Northwest as potential candidates for potential new drug prospects.

Methods

Data were collected in 2016 at four study sites along a North-South transect from southern Oregon to central Washington (Figure 1). The study sites are part of an unrelated, but ongoing, study called the Douglas-fir Seed Source Movement Trial (SSMT), a long-term, large-scale reciprocal provenance study of Douglas-fir [16] (Table 1). The sites vary in both elevation (185-860 m) and mean annual precipitation (250-1,575 mm/year). The trial includes 120 open-pollinated families of coast Douglas-fir that were grown from seed collected from populations in western Washington and Oregon and northern California and planted prior to the 2009 growing season (Table 2).

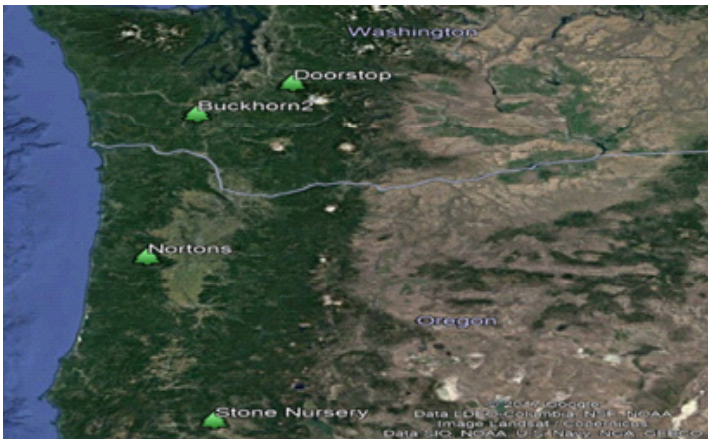

Figure 1: Locations of four study sites, part of the Douglas-fir Seed Source Movement Trials (SSMT), a long-term, large-scale reciprocal provenance study of Douglas-fir. Regions vary in characteristics such as annual precipitation and elevation, and are meant to represent large-scale variations in geographic and climatic conditions experienced by Douglas-fir in the Pacific Northwest.

Site/State	Region	Code	Elevation (m)	MAT* (°C)	MAP (mm)
Nortons (OR)	Coast North	ORCstN	185	10	1198
Stone Nursery (OR)	Cascade Low	ORCasL	415	12	250
Buckhorn2 (OR)	Cascade Low	WACasL	240	10	1149
Doorstop (WA)	Cascade High	WACasH	860	8	1575
*MAT - Mean Annual Temperature, MAP - Mean Annual Precipitation.					

Table 1: Climate variables associated with seed sources (top) and planting sites (bottom) of the Seed Source Movement Trial (SSMT), a long-term, large-scale reciprocal provenance study of Douglas-fir.

A nested sampling design was used to collect Douglas-fir branch lets from three different seed sources at each site (Table 2). Within each site, one seed source represents the local population, while the remaining two seed sources represent non-local populations. Non-local seed sources were chosen randomly and without replacement. The second non-local seed source at Doorstop was omitted, as it was a duplicate of the local seed source at Stone Nursery. Therefore, analyses focuses on the 11 remaining seed sources.

Planting Site	Local Seed Source	Non-Local Seed Sources	
Buckhorn2	WACasL	WACst	ORCasL
Doorstop	WACasH	CAKla	(ORSisL—OMITTED)
Nortons	ORCstN	ORCstS	ORCasH
Stone Nursery	ORSisL	CACst	CASierra

Table 2: Seed sources sampled at each site. Planting sites are part of the Seed Source Movement Trial (SSMT), a long-term, large-scale reciprocal provenance study of Douglas-fir.

Two to four blocks were sampled at each site, depending on the availability of living samples. For several seed sources, at least one block contained no living trees of that source and could not be sampled. Within each section, one haphazardly chosen tree and one branchlet were sampled. The branchlet position on each tree varied both in vertical position (high, medium, or low) and cardinal direction (N, S, E, W). Vertical and directional variations were based on branchlet availability within reach. Branchlets were stored at -20 ˚C prior to analysis. Thirty-seven total branchlets were collected across all sites.

Traditional Plate Culturing

Needles were removed from each branchlet and surface-sterilized in 2% bleach for 5 minutes, then rinsed in deionized water and transferred to a sterile 15 mL collection tube [17]. Needles were randomly chosen from the collection tube for sterile plating, cut into 5 or more pieces, and placed onto 1.5% Potato Dextrose Agar (PDA) [Difco™ (Becton, Dickinson and Company, Sparks, MD, USA)]. Each plate was evenly divided into three observation units, with one sectioned needle from the same branchlet sample placed in each unit. For each seed source, 5 PDA plates were used, for a total of 15 needles from each seed source. Plates were visually observed daily for evidence of fungal growth. Newly observed growth was immediately transferred to a 1.5% PDA plate to isolate pure cultures. After at least fourteen days of culturing, isolates were examined and grouped based on colony morphology. One representative of each morphogroup was haphazardly chosen for sequencing; however, a conservative approach was applied, where morphological uncertainties were sequenced to prevent species omission. Pure fungal tissue was collected from potato dextrose broth incubations for molecular analysis. Surface sterilization effectiveness was tested using a sub-sample from stored collection tubes and methods described in [17]. No plates exhibited growth originating from imprinted areas, suggesting surface sterilization was effective.

DNA Extraction

Isolate DNA was extracted using a DNeasy Plant Mini Kit® (QIAgen, Valencia, CA, USA), according to standard protocol. Fourteen isolates could not be extracted due to slow or absent growth in liquid media. DNA was extracted from these isolates using a modified Cetyl Trimethylammonium Bromide (CTAB) DNA extraction protocol [18]. Isolate extracts underwent DNA amplification via Polymerase Chain Reaction (PCR) [19]. Following PCR, amplicons were checked on a 1.5% agarose gel stained with the Nucleic Acid Staining Solution RedSafe™ (iNtRON Biotechnology, South Korea) for a single, target fungal band. PCR product was purified with Exo-Sap™ PCR product cleanup kit (Affymetrix, Santa Clara, CA, USA) following manufacturer protocol. Purified product was submitted to the Center for Genome Research and Biocomputing, OSU, where Sanger sequencing was performed with an ABI 3730 capillary sequence machine (Life Technologies, Grand Island, NY, USA). Completed sequences were assembled into contigs using Sequencher 4.9 and fungal identities were determined using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi Accessed 8 August 2017).

Results

Of the 555 Douglas-fir needles sampled, 318 isolates (221 identified) from 24 identified families and one class were cultured from 215 needles (Table 3). 97 isolates were unidentified and grouped into an unknown category for percentage calculations. In total, 46 unique isolates from 39 taxa were identified from needles with *Xylariaceae* accounting for nearly 30% of infections (Table 4). Sixty-seven isolates were successfully sequenced but could not be identified to the genus level by DNA sequences because no ITS sequences matching with high similarity in the sequence database could be found. Thirty of the unidentified cultures were unsuccessful in amplification after multiple attempts.

Species	Frequency	Accession Number(s)
Anthostomella conorum[1]	6	EU552099, KT149745
Anthostomella pinea	1	KJ406991
Apodus deciduus	1	AY681199
Aposphaeria corallinolutea	1	KY554202
Aureobasidium pullulans	4	LC277152, JX188099
Aureobasidium sp.	1	HF674760
Cladosporium perangustum	1	MF303712
Claussenomyces sp.[1]	2	KT264343
Clypeosphaeria mamillana[2]	4	KT949898
Clypeosphaeria sp.	1	JQ341099
Coniochaeta hoffmannii	1	KX869937
Crustomyces subabruptus[1]	24	KP814558
Cryptococcus sp.	1	KM216339
Cryptostroma corticale[2]	3	KR870994
Diaporthe sp.[1]	2	LC041016
Dothideomycetes sp.	4	KP990991
Geopyxis rehmii	2	KU932461
Graphostroma sp.[1,2]	13	EU715682
Helicoon fuscosporum	2	EF029203
Hormonemia sp.	2	AF013225
Hypoxylon rubiginosum[2]	21	AY787708
Lecythophora sp.[1,2]	7	KX096678, GU062252, AY219880
Melanomma pulvis-pyrius	2	KY189979
Nemania serpens	1	KU141386
Nemania sp.	1	HM123573
Ophiognomonia alni-viridis	1	JF514848
Penicillium glabrum[1,2]	18	KU847873, KY318471, KX099660, KX609402, KU847870
Perusta inaequalis[1]	2	NR_144958
Pezizomycetes sp.[1]	3	KX909069, KJ508333
Phaeomoniella zymoides	2	GQ154600
Phomatospora biseriata	1	KX549454
Podospora sp.	1	AM262361

Preussia bipartis[2]	3	GQ203774
Rhabdocline parkeri[1]	8	AF462428, AF462427
Rosellinia quercina	3	AB017661
Rosellinia sp.[1]	3	KT264658
Sordariales sp.[1]	2	FN548158
Sordariomycetes sp.[1,2]	9	KT264524, KX611007, KX611549, JQ759589, GQ153043, KP992078, GQ153206
Sporormiaceae sp.	1	KX611024
Sydowia polyspora	1	KP152486
Talaromyces ruber[1]	2	JX965239
Taphrina communis[1]	13	AF492088
Taphrina veronaerambellii	1	NR_111148
Wallemia sebi	1	KX911858
Xylaria hypoxylon[1,2]	35	KY204024, KX096696, GU300096
Xylaria sp.[2]	3	AB465207
Unidentified	97	
[1]Common among sites. [2]Common among non/local seed sources within sites.		

Table 3: Taxa and frequency of endophyte species cultured and sequenced from 185 Douglas-fir needles from four sites in Oregon and Washington. The number of endophytes successfully sequenced totals 221 out of 318 isolates.

Site Name	Type	Seed Source	Isolates	Needles Cultured	% Infection Rate
Buckhorn2	Non-local	WACst	33	60	55%
Buckhorn2	Non-local	ORCasL	18	30	60%
Buckhorn2	Local	WACasL	22	45	49%
Doorstop	Non-local	CAKla	22	45	49%
Doorstop	Local	WACasH	15	45	33%
Nortons	Non-local	ORCstS	15	60	25%
Nortons	Non-local	ORCasH	15	45	33%
Nortons	Local	ORCstN	22	45	49%
Stone	Non-local	CACst	17	60	28%
Stone	Non-local	CASierra	33	60	55%
Stone	Local	ORSisL	3	60	5%
		Total:	**215**	**555**	**39%**

Table 4: Endophyte infection rates for each seed source sampled at four sites in Oregon and Washington.

Discussion

Infection rates for Douglas-fir needles in this study were well below infection rates reported in Carroll and Carroll (1978). Wet site infection rates averaged just under 27% (44.5% vs. 71.3%). Plating methods and sterilization methods were similar for both studies. However, infection rate in the first study was calculated as total number of infected needle segments divided by total number of incubated segments whereas this study calculated infection rate as a binomial ratio of number of total needles infected divided by total needles incubated. Because the infected needles had a ratio of 1.48 infections per needle (318/215), a more similar infection rate of 65.9% could be calculated for the current study. Additionally, differences might be explained by the age difference in the sample trees. The primary site for the earlier study [11] is the H.J. Andrews Experimental forest and we surmise that the age of the sample trees was much older than the trees in this study (8-9 years of age post planting).

Comparisons of species lists between the current study and the earlier study [11] are not applicable since genomic technologies (PCR and BLAST for example) were not available and the earlier study had to rely on morphological identification. Carroll and Carroll (1978) noted the difficulties in obtaining sporulation for species identification relying on code numbers. In addition, only those fungi that accounted for at least 1% of total infection were listed and they noted that may have constituted a majority of species (80-90%). The present study identified all occurrences of species regardless of percent infection rate (Table 3).

Endophytic fungi reside inter or intracellularly within their hosts in an environment of host resistance. Additionally, they often exist alongside other microbes (fungi and/or bacteria) that are involved in defensive relationships among themselves. Therefore, it is not surprising that the majority of identified endophytes show a propensity to produce bioactive compounds. Because these relationships are generally asymptomatic, we can surmise that the relationships involved are at some type of biological standstill, or what has been termed, a mutual balanced antagonism [20].

Nearly 70% of endophyte species (32 of 46) identified in this study have been shown through the literature to produce bioactive compounds. Several of the remaining 14 species are either suspected of producing bio actives, or have not yet been bio-assayed for bioactive production. Typically, compounds produced are either antifungal or are active as resistance compounds against plant-active alkaloid defenses. Additionally, with the fungal microbiome still largely unidentified, we are continuing to work with the successfully sequenced but unidentified cultures.

It is important to note that complexities in the plant-fungal interaction can include both a physical ability to produce metabolites based on molecular identification of host/pathogen as well as environmental variables affecting metabolite production. For example, this study has focused on young-age Douglas-fir. However, conifers are known to produce a very diverse array of terpenoids [21]. Host-pathogen responses are well known to be dependent upon the molecular recognition of key markers to initiate either host-response or pathogen-response to production of bioactive compounds. This can result in changes to the fungal endophyte community structure between conifer species. Thus, we would be reasonable to expect that western hemlock (*Tsuga heterophylla*) might not only have a different fungal endophyte community structure, but also be susceptible to fungal endophyte pathogens that produce unidentified molecular markers that fail to initiate host defense responses.

Additionally, within species variation of metabolite concentrations have been shown to correlate with environmental variables [22]. For example, elevated CO_2 levels significantly reduced levels of 50% of the main monoterpene compounds in needles within Douglas-fir by up to 52%, Additionally, elevated temperatures were correlated with a 64% reduction in total monoterpene production. Thus, elevated temperatures coupled to elevated CO_2 levels could be expected to result in significant changes to fungal endophyte communities as well as overall forest health conditions.

Species within the *Xylariaceae* comprise one of the largest and most ubiquitous families in the Ascomycota with approximately 85 genera and 1340 species [23]. While generally considered saprophytic, a number of species have been reported to be endophytic [24] and/or pathogenic [25]. Moreover, a number of new compounds have been isolated from *Xylariaceae* that demonstrate antimicrobial or anticancer bioactivity, particularly endophytic *Xylaria* [26,27]. For example, the most prevalent *Xylaria* isolated from this study, *Xylaria hypoxylon*, has been found to be a rich source of several cytochalasins, noted metabolites exhibiting marked cytostatic effects on mammalian cells in tissue culture, inhibition of HIV-1 protease 2, as well as antibiotic and antitumor activities [28]. Nearly 30% of the isolations (97/318) from this study comprised 15 endophytic genera or species from the *Xylariaceae* (Table 4). Given the wide distribution of the family, the *Xylariaceae* may be a very important bioprospecting source for screening new pharmaceutical and other natural products.

A number of other species isolated in this study show promise as sources of new or novel drug discovery. Among these, *Pennicilium glabrum* and *Talaromyces ruber* (*Trichocomaceae*) comprised just over 6% of the identified isolations. The genus is generally considered one of the most common among fungi and is demonstrated to have a high affinity for production of bioactive metabolite [29] making it highly desirable for consideration of bioprospecting particularly in underrepresented habitats such as that found in forests.

Aureobasidium is another widely distributed genus of approximately 15 recognized species. *A. pullans* is particularly important as different strains are capable of producing amylase, proteinase, lipase, cellulase, xylanase, mannanase, transferases, pullulan, siderophore, and single-cell proteins [30]. Because the different strains have the capability of numerous bio product formulations it is an important genera to continue to bio prospect. Additionally, as the other species of the genus are not as well understood, they are an excellent candidate for further bioprospecting, particularly in less understood bio systems.

The genus complex *Coniochaeta* (*Lecythophora* anamorph stage) is particularly interesting as it is one of the few endophytes to produce bioactive antifungal compounds in vitro in concert with the host plant *Smallanthus sonchifolius.* Twelve fatty-acid antifungals were derived from autotrophic tissue cultures and shown to be affective against target phytopathogenic fungi in the genus *Colletotrichum* [31]. Additionally, bioactive compounds of *Lecythophora* mutabilis have been reported to have human pathogenic potential [32].

Cladosporium perangustum is a species reported to be found in a dominant frequency [33] in part due to possible antibiotic metabolite production as well as antitumor/antifungal compounds capable of resistance to plant-activated defense alkaloids [34].

Studies on bioactive compounds of land-based *Graphostroma* sp. could not be found, however the deep sea species, *Graphostroma* sp. has yielded a series of nine guanines that are shown to be highly inhibitory in the control of anti-inflamation [35]. Cultures of *Dothediomycetes* sp. isolated from the medicinal plant *Tylophora indica* has been shown to produce antifungal bioactives with a high inhibition rate against phytopathogens *Fusarium oxysporum* and *Sclerotinia sclerotiorum* [36]. The compound rugulosin has been isolated as the insect toxin produced by the endophyte Homonema deltoids (*Sydowia polyspora* telomorph) [37]. Two new benzopyranones have been isolated from *Diaporthe* sp. and have been shown to be inhibitory to a virulent strain of *Mycobacterium tuberculosis* H37 Rv [38]. Bioactive compounds have been additionally reported for *Pezizomycetes* sp., *Phaeomoniella zymoides, Podospora* sp., *Rhabdocline parkeri, Rosellinia quercina, Sordariomycetes* sp*, Sporormiaceae* sp., and *Wallemia sebi*.

Conclusions

We report on a number of pharmaceutically important fungal endophytes not previously identified in Douglas-fir. The microbiome of forests is still fairly unknown and is particularly open for discovery. Additionally, the potential for bioprospecting forest endophytes for new and unique pharmaceuticals and other natural products is certainly important, particularly in light of the frequency of endophytes capable of producing bioactive compounds shown in this and other studies. The renewed interest in bioprospecting the microbiome of forests is exciting in light of the de-emphasis on combinatorial chemistry coupled to the limited knowledge of fungal species in whole. Certainly, there are difficulties to overcome. For example, limitations include the problem of orphan biosynthetic pathways and *in vitro* processes for bioassay, however strategies and emerging technologies exist for dealing with these.

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