Final Report for MDARD Horticulture Fund

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Final report for the 2018 Horticulture Fund. Project Title: Biocontrol of spruce decline -A comparison of interactions between *Diaporthe* spp. and fungal endophytes isolated from Colorado blue spruce.

In May 2018, a research project was initiated to investigate potential biological control agents of the *Diaporthe* spp. involved in spruce decline. We hypothesized that asymptomatic Colorado blue spruce (CBS) trees in areas with high spruce decline pressure have sapwood, cambium or inner bark colonized by antagonistic organisms inhibiting the pathogen's ability to cause cankers and there were different endophytic fungi composition between symptomatic and asymptomatic CBS trees. This project was focused on culturable fungal endophytes and their interaction with CBS trees. The main objectives are 1) to isolate and identify endophytic fungi from asymptomatic and symptomatic CBS trees in areas with high spruce decline pressure, 2) to characterize culturable endophytic fungal communities between the symptomatic and asymptomatic CBS trees, 3) to screen and compare the *in vitro* antagonistic ability of the endophytic fungi against *Diaporthe* spp., and 4) to evaluate *in planta* interactions between host, endophytic fungi and pathogen.

- Activity 1: Isolation and identification of endophytic fungi.
- Activity 2: Characterization of endophytic fungal community composition.
- Activity 3: *In vitro* bioassay tests for antagonistic activity.
- Activity 4: *In planta* host colonization and disease challenge assays.

To date, we have completed Activity 1, 2 and 3, and have been conducting Activity 4 where we are collecting final data for the *in planta* assays. Endophytic fungi have been isolated from symptomatic and asymptomatic Colorado blue spruce trees in North, West and East of the Lower Peninsula of Michigan. We have completed identification of the fungi using molecular barcoding and characterization of the fungal community composition of symptomatic and asymptomatic CBS trees. Antagonistic activities of the endophytic fungi were tested on dual culture and culture filtrate assays. Endophytic fungi were selected and used for the plant interaction assay after antagonistic activities are confirmed *in vitro*. We found promising endophytic fungi, *Aspergillus unguis*, *Epicoccum nigrum*, and *Talaromyces* sp., producing bioactive antifungal metabolites in the culture medium that inhibit the growth of pathogen *Diaporthe* spp. Results of the activities and financial expenses for the project are presented in this final report.

Results of Activity 1 and 2: Isolation and identification of endophytic fungi and Characterization of endophytic fungal community composition.

We have focused on isolation of culturable endophytic fungi and identification using morpho-grouping and molecular barcoding analysis. We have collected 4 sample sets of symptomatic and asymptomatic CBS trees from Northern (Cadillac), Western (Port Sheldon) and Eastern (Brighton) of the Lower Peninsula of Michigan (Fig. 1). Woodchips with vascular tissues were processed from 3 randomly selected branches of each CBS trees. Surface sterilized woodchips were place on 2% malt extract media and incubated at 20°C until hyphal growth was observed (Fig. 2). All hyphal growth from each wood chip were sub-cultured and initially grouped to morpho-types based on colony or vegetative characteristics and then representatives from each group were identified via rDNA sequences for the internal transcribed spacer (ITS) region using primers ITS1f and ITS4 by NCBI nBLAST searches.

Fifty-three endophytic fungal taxa were recovered from symptomatic and asymptomatic CBS trees from the 4 sets of samples: 8 and 22 fungal isolates were isolated from asymptomatic or symptomatic CBS trees, respectively, where 9 fungi were shared (Table 1). *Paraconiothyrium brasiliense* (31.6 and 22.3 %, respectively) is most common in both CBS trees. *Epicoccum nigrum* (14 %) is the second most common in asymptomatic trees, and *Diaporthe eres* (17 %) in symptomatic trees (Table 1). Several different *Diaporthe* spp. were involved in the group of endophytes isolated from the symptomatic CBS trees.

Fig. 1. A sample set of asymptomatic and adjacent asymptomatic CBS trees

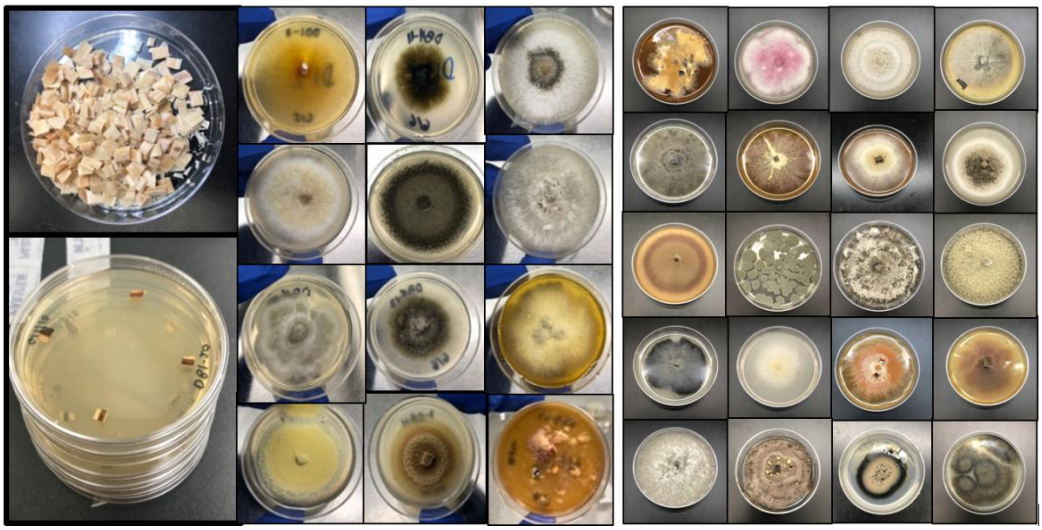

Fig. 2. Isolation of endophytic fungi and morpho-type grouping colonized in symptomatic and asymptomatic CBS trees

Table 1. Molecular identification of endophytic fungi isolated from symptomatic and asymptomatic CBS trees in Michigan.

Taxon	NCBI reference	Similarity (%)	No. of isolate	Antibiosis [a]	Frequency (%)	
					Asymptomatic	Symptomatic
Alanphillipsia aloetica	KF777139	94.3	1		0.0	9.5
Alternaria alternata	MH578598	100	3		8.8	1.8
Alternaria sp.	MK461082	100	1		0.0	0.7
Aspergillus amoenus	LN898664	99.8	1		7.0	0.7
Aspergillus unguis	KU866670	99.9	1	+++	1.8	0.0
Biscogniauxia cinereolilacina	MH862567	99.1	1		1.8	0.0
Chaunopycnis pustulata	AF389189	99	1		0.0	0.4
Cladosporium aerium	MF472897	100	1		1.8	0.0
Cladosporium chasmanthicola	NR152307	100	1		1.8	0.0
Cladosporium herbarum	MH047193	100	1		0.0	2.1
Claussenomyces sp.	KY633581	100	1		0.0	0.7
Cytospora carbonacea	MG879505	98.6	1		0.0	1.1
Cytospora sp.	MG719648	97.6	1	++	0.0	1.1
Diaporthe cotoneastri	KC145903	100	1		0.0	0.7
Diaporthe eres	KP903572	99.3	4		5.3	17.0
Diaporthe nobilis	KJ609006	99.7	1		1.8	0.4
Diaporthe sp.	KU712214	98.8	1		0.0	3.5
Diplodia seriata	MK012553	99.8	1		0.0	6.4
Dothideomycetes sp.	GU595039	96.8	2		3.5	0.4
Epicoccum nigrum	HQ115657	100	5	+++	14.0	2.1
Exophiala bergeri	MH857080	98.2	1		0.0	0.4
Fungal endophyte 1	EF419935	99.8	1		5.3	2.1
Fungal endophyte 2	KR015187	95	1		0.0	0.7
Fungal endophyte 3	EU686080	92.7	1		3.5	0.0
Fusarium acuminatum	LT970802	100	1		0.0	1.1
Helminthosporium velutinum	AB551948	97.2	1		3.5	1.4
Hysterobrevium constrictum	KY496739	97.2	1		0.0	0.4
Lophiostoma macrostomum	EU552140	99.7	1		0.0	0.4

Microdochium bolleyi	AM502261	95.6	1		0.0	0.7
Mucor brunneogriseus	MH856086	99.2	1		0.0	0.4
Mucor hiemalis	HM172811	99.8	1		1.8	0.0
Myrothecium verrucaria	EF211127	99	1		0.0	11.0
Paraconiothyrium brasiliense	JF439492	99.8	4		31.6	22.3
Penicillium expansum	MH879835	99.8	1		1.8	0.4
Phaeobotryosphaeria porosa	KF766210	95	1		0.0	7.1
Pleosporales sp.	MH497576	98.9	1	+	0.0	0.7
Rhizosphaera kalkhoffii	AF013232	99.5	1		1.8	0.0
Sarea resinae	MH857111	98.8	1		0.0	0.4
Talaromyces sp.	KU556530	99.8	1	++	1.7	0.0
Valsa pini	JX438617	99.1	1	++	0.0	2.5
Wojnowiciella dactylidis	LT990661	99.7	1	+	3.5	0.0
Total			53		100	100

[a] inhibition zone: + (weak), ++ (intermediate) and +++ (strong)

Results of Activity 3: *In vitro* bioassay tests for antagonistic activity

We have tested endophytic fungi isolated from the 4 sets of samples using dual culture assays to evaluate the *in vitro* antagonistic activity of endophytic fungi isolates by placing hyphal agar plugs (6 mm) of *Diaporthe* spp. and endophytic fungi in the Petri dishes containing 2% malt extract agar (MEA) media (Fig. 3). Interactions were evaluated as antibiosis (characterized by the presence of a stable inhibition zone between the endophyte and pathogen), substrate competition (greater growth of the endophyte than the pathogen), neutral (similar growth of the endophyte and pathogen) or mycoparasitism (parasitism of hyphae of the pathogen by the endophyte). The antibiosis and competitive interactions were evaluated by measuring the pathogens reduced radial growth: Inhibition (%) = [(R-r)/R×100] where, R represents the average radius of mycelial growth on control plates and the r is the radial growth of the pathogen with the endophyte in dual culture.

Out of the 53 endophytic fungi isolated, 14 showed *in vitro* antagonism of the *Diaporth*e spp. in dual culture assays, showing the growth inhibition rates up to 40-50 % and 30-40% approximately against *Diaporthe vadumcancri* prov. nom.[1] and *Diaporthe eres*, respectively. Among them, *Aspergillus unguis*, *Cytospora* spp., *Epicoccum nigrum*, *Pleosporales* spp. *Talaromyces* spp. *Valsa pini*, and *Wojnowiciella dactylidis* exhibited antibiosis with a stable inhibition zone (Table 1). Inhibition zone is considered as antibiosis characteristics of fungal endophytes producing unique bioactive metabolites like antifungal compounds.

Consequently, we have tested the activity of the liquid culture filtrate of endophyte isolates above after culturing the fungi in the 2% malt extract broth media (Fig. 4). Each filtrate was mixed with the MEA media (50%) where a mycelial agar plug of *Diaporthe* spp. were inoculated. The growth of *Diaporthe vadumcancri* prov. nom. and *D. eres* were significantly inhibited by amendment of the liquid culture filtrate 7 days after incubation at room temperature (Fig. 5). *Aspergillus unguis* (97%), *Epicoccum nigrum* (58-72%) and *Talaromyces* sp. (61%) isolated from asymptomatic trees showed mycelial growth inhibition of *Diaporthe* spp. in culture filtrate assays (Fig. 5 and 6). *Cytospora* spp. showed inhibitory activity against *Diaporthe* spp. but was not considered in future studies because *Cytospora* spp. is a known pathogen of spruce. *Epicoccum nigrum* isolates from asymptomatic CBS trees were significantly more effective in inhibiting *Diaporthe* spp. growth than *E. nigrum* isolates from symptomatic trees. Therefore,

endophytic fungi in asymptomatic CBS trees seems to play an important role in hindering symptom development of spruce decline.

Fig. 3. Dual culture assays for testing antagonistic activities of endophytic fungi against *Diaporthe* spp.

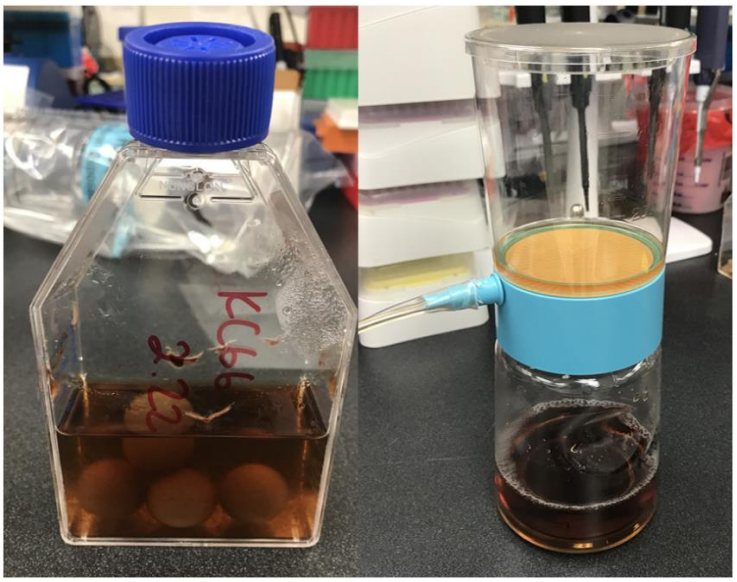

Fig. 4. Culture filtrate production of endophytic fungi showing *in vitro* antagonistic activities

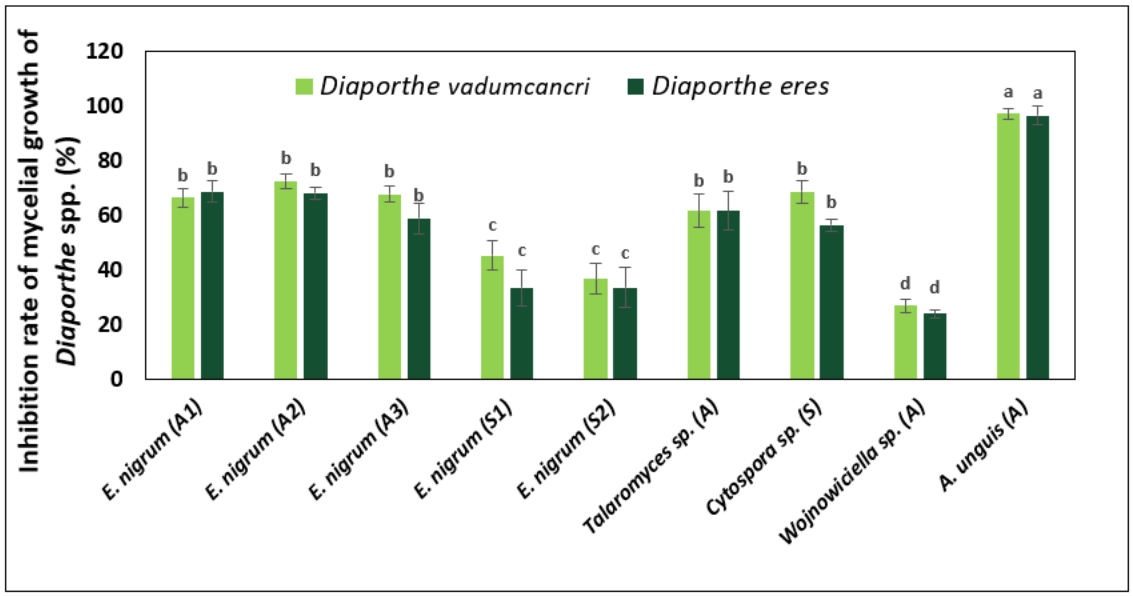

Fig. 5. Mean percentage of growth inhibition of *Diaporthe* spp. in the culture filtrate assayed 5 days after incubation using various endophytic fungal isolates (A: asymptomatic and S: symptomatic). Bars represent the standard error of the mean (n=5). Bars labeled with same letter are not significantly different among the treatments and experiments at $P < 0.05$ using the Tukey's honestly significant difference test.

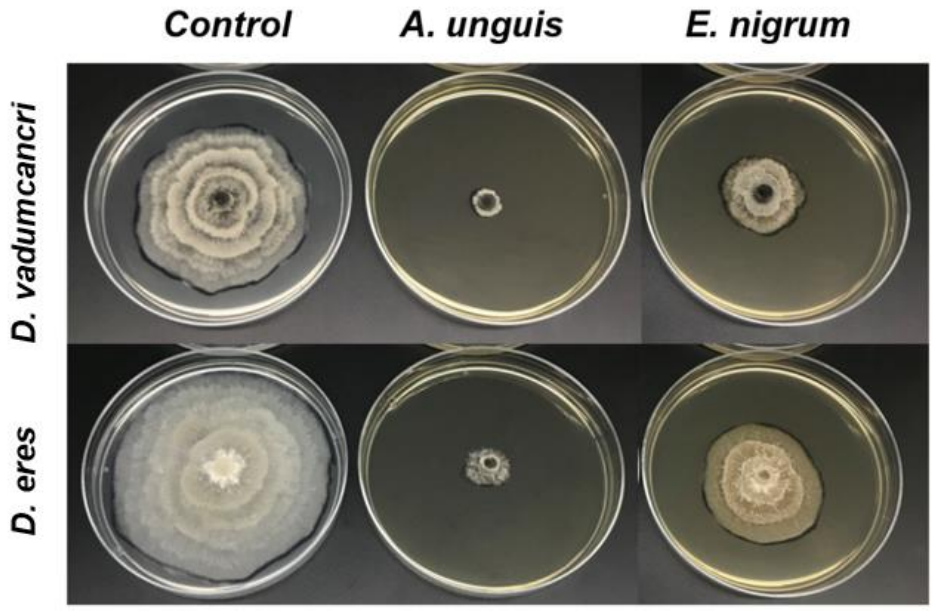

Fig. 6. Mycelial growth inhibition of *Diaporthe* spp. on media amended with culture filtrates produced by *Aspergillus unguis* and *Epicoccum nigrum*.

Progress on Activity 4: *In planta* host colonization and disease challenge assays

Endophytic fungal candidates have been assessed for the *in planta* greenhouse experiments to evaluate their efficacy within CBS trees as a biological control of *Diaporthe* spp. associated with spruce decline (Fig. 7.). Greenhouse experiments were performed at the Tree Research Center in Michigan State University. Seedlings (4 years, 2-2) were transplanted in plastic pots (2 gallon) filled with soilless media (media mix of peat, perlite, wetting agents and starting nutrients; Suremix perlite, Michigan Grower Products, Inc., Galesburg, MI, USA). The inoculum of *Diaporthe* spp. and endophytic fungal isolates were prepared on PDA medium. The plants were inoculated by placing a mycelial agar plug (6 mm) facing on the cambial region under the bark 2-3 (endophytes) and 7-8 cm (*Diaporthe* spp.) above the soil surface where lateral incisions were made with a sterile scalpel blade. *Aspergillus unguis*, *Epicoccum nigrum.* and dual inoculation of them were pre- and post-inoculated in CBS trees to test preventive and curative inhibition effects on canker development caused by *Diaporthe* spp. The controls were inoculated with PDA without mycelium. The treatments were arranged in a randomized complete block design with 5 replications. The canker lesion area will be measured after 4 weeks from the *Diaporthe* spp. inoculations.

Fig. 7. Greenhouse experiment to test colonization of endophytes and disease challenge assays *in planta*.

Greenhouse experiments will be finished in October 2019 then results from this project will be submitted in a peer reviewed journal. Findings were presented in research conferences and submitted to be published in the *Phytopathology* journal.

Shin, Keumchul., Medina Mora, C., Sakalidis, M.L. 2019. Evaluation of endophytic fungi isolated from Colorado blue spruce (*Picea pungens*) in Michigan for biological control of spruce decline caused by *Diaporthe* spp. To be submitted to *Plant Disease* Nov. 2019.

Sakalidis, M.L, Medina-Mora, C.M., Shin, K., Fulbright D.W. Characterization of *Diaporthe* spp. associated with spruce decline on Colorado blue spruce (*Picea pungens*) in Michigan. Phytopathology. Reviewed and revisions in progress as of Sept. 6th 2019.

Sakalidis, M.L., Medina Mora, C, Shin, K, Fulbright. D.W. 2019. Two species of *Diaporthe* cause needle-loss, branch dieback and cankers on Colorado blue spruce (*Picea pungens*) in Michigan, American Phytopathological Society Meeting, Cleveland, Ohio, USA.

Shin, K., Medina Mora, C., **Sakalidis, M.L.** 2019. Biocontrol of spruce decline: Comparison of interactions between *Diaporthe* spp. and fungal endophytes from Colorado blue spruce (*Picea pungens*), American Phytopathological Society Meeting, Cleveland, Ohio, USA.