
Healthy grapevines


Declining grapevines

Molecular Phylogenetics of Grapevine Decline Fungi from Pennsylvania and New York.

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Introduction

Twenty-seven vineyard sites including more than 500 acres of vines were examined over three growing seasons (2001-03) to quantify the extent of vine decline and to isolate fungi from declining vineyards. Petri disease caused by *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* typically results in the decline or slow-dieback of young vines. Three hundred sixty-two fungal isolates were recovered from vines exhibiting decline symptoms in Pennsylvania (PA) and New York (NY). Twenty-nine percent of the fungi isolated were those associated with Petri disease. *Phaeoacremonium inflatipes* and *P. parasiticum* were not isolated from declining vines in PA or NY. Twenty-four percent of the fungi isolated were Phialophora-like, referable to the genera *Cadophora*, *Harpophora*, and *Phialophora* s.s. Twenty-four percent were pycnidial fungi, with 8% identified to the genus *Phomopsis* (**See Fig. 1**). Additional survey work and extension visits were made over 2003-2004 and 188 additional isolates added to the culture collection from PA, NY and Virginia establishing a culture collection of 550 isolates. There is an ongoing effort in our laboratory to sequence the ITS rDNA region from all isolates in the culture collection. To date, the ITS rDNA gene region has been sequenced for 161 of the 550 isolates. Data obtained from the ITS rDNA region will be used to determine target strains for further phylogenetic study, including phylogenetic analyses based on other gene regions. The culture collection at Penn State can be used for ecological and plant pathological studies, generation of sequence data that can be used for identification of potentially new species, and for developing pathogen detection systems (real-time PCR).

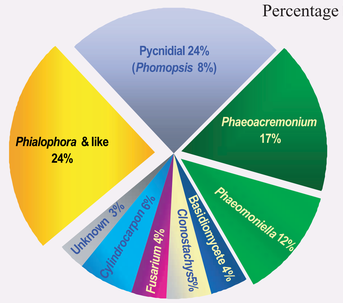


Fig. 1. Genera isolated from vine decline survey (2001-2003). Percentage based on 362 isolates.

Establishing Biological Characters with Taxonomic Significance

Phaeoacremonium spp. and *Phaeomoniella chlamydospora* in association with several basidiomycetes are implicated as associated agents of a disease complex referred to as esca or black measles that causes a decline of mature grapevines. Mugnai et al. (1999) showed that Petri disease fungi involved in esca produced ligninolytic enymes in the peroxidase family. Two white rot basidiomycetes have been found associated with esca, *Fomitiporia punctata* and *Stereum hirsutum*. White rot fungi produce four families of oxidative enzymes, lignin peroxidase(LiP), laccase, manganese-independent peroxidase (MIP), and manganese-dependent peroxidase (MnP) (Moreira et al., 1999) MnP is the most common peroxidase encountered in nature (De Jong et al., 1994). MnP oxidizes Mn(II) to Mn (III) which acts as a diffusible agent that can oxidize lignin moieties (Glenn et al., 1986). *Stereum hirsutum* has an MnP that can be used for the bio-bleaching of kraft pulp (Moreira et al., 1999). It is possible to assay for Manganese-dependent peroxidase using Mn-amended agar plates because oxidized manganese will form a brown pigment or black crystals of Mn dioxide in vitro (Otrosina and Illman, 1994).

1. Can esca-related fungi, specifically *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., oxidize manganese *in vitro*?

See Table 1.

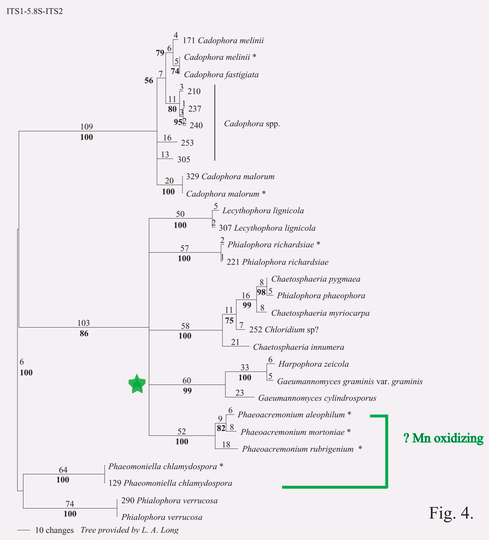


Fig. 4.

* = *ex-type* ITS1-5.8S-ITS4 sequence. *Gaeumannomyces* spp. are know to oxidize Mn. Don Huber (Pers. Com. viz. seminar)

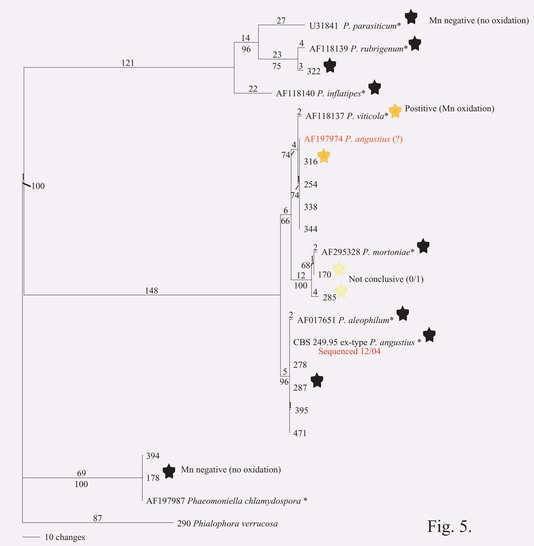


Fig. 5.

Phylogram showing 1 of 6 most parsimonious trees based on ITS1-5.8S-ITS4 sequence data (10 radom addition sequence replicates). The bootstrap values (500 replicates, 10 radnom addition sequence replicates) are beneath branches. * = *ex-type* ITS1-5.8S-ITS4 sequence.

2. Can Manganese-amended media be used to separate species based on ability to oxidize Mn?

See Figs. 2--3.

Table 1. Differential response of esca-related fungi to manganese sulfate-amended media.

Fungal Species	Control (no Mn)	22 PPM	80 PPM	100 PPM	300 PPM	1000 PPM	2000 PPM	3000 PPM	4000 PPM	5000 PPM	6000 PPM
Stereum hirsutum HHB-4401-Sp	0	1	1	1	1	1	1	1	1	1	1
Fomitiporia punctata FP-133888-3p	0	0/1	0/1	0/1	RG	RG	No Growth				
Phaeoacremonium angustius CBS 249.95	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium angustius (316)	0	1	1	1	1	1	1	1	1	1	1
Phaeoacremonium rubrigenum CBS 498.94	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium rubrigenum (322)	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium aleophilum CBS 246.91	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium aleophilum (287)	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium viticola CBS 101738	0	1	1	1	1	1	1	1	1	1	1
Phaeoacremonium inflatipes CBS 391.71	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium parasiticum CBS 860.73	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium mortoniae CBS 101585	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium mortoniae (170)	0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Phaeoacremonium mortoniae (285)	0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Phaeomoniella chlamydospora (178)	0	0	0	0	0	0	0	RG	RG	No Growth	

Indicates positive reaction.

Reduced growth (RG) or no growth.

Not conclusive

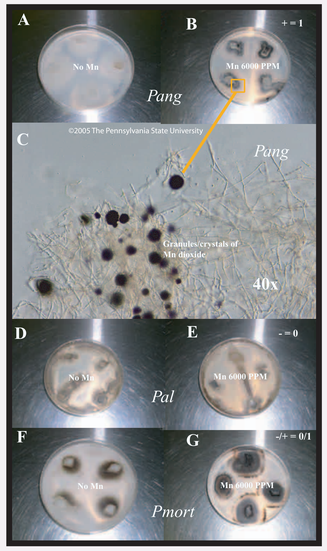
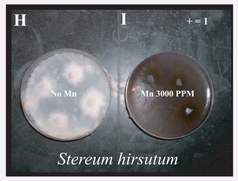


Fig. 2.



Stereum hirsutum has a well characterized Mn-dependent peroxidase involved in lignin degradation.

Note the strong reaction in the assay.

Fig. 3.

CHEMISTRY

Manganese sulfate-amended Potato Dextrose Agar (PDA) at various MnSO4 (Fw 151) concentrations (Table 1) creates an environment in which Mn(II) is available for enzymatic oxidation to Mn(III). Mn(II) is oxidized by manganese-dependent peroxidase (MnP) an enzyme involved in lignin degradation resulting in Mn(III) and can be visualized by formation of a rust color in Manganese-amended PDA (See Fig 3). In some cases, autoxidation of Mn(III) will form insoluble black crystals of Mn dioxide (See Fig 2, C). Therefore, the oxidation of manganese can be assayed *in vitro* (qualitative) **and potentially used as a diagnostic character for some taxa.**

RESULTS

A--C.) *Phaeoacremonium angustius* culture 316 (Pang). Crystals are visible in PDA amended with Mn sulfate, but not control (no Mn sulfate). No brown pigment production. Assay is scored as positive for Mn oxidation (1).

D--E.) *Phaeoacremonium aleophilum* culture 287 (Pal). No pigment production or crystals are visible in PDA amended with Mn sulfate. Assay is scored as negative for Mn oxidation (0).

F--G.) *Phaeoacremonium mortoniae* culture 170 (Pmort). Brown rust colored reaction near hyphal tips in PDA ammended with Mn sulfate but no crystals of Mn dioxide visible. This reaction is scored as -/+ (0/1) result because cultures also produced a brown pigment in the control (PDA with no Mn sulfate). However in the control, the pigment was not produced near the hyphal tips. The ex-type culture of *P. mortoniae* did not produce pigment near the hyphal tips.

H--I.) *Stereum hirsutum* culture HHB-4401-Sp. Rust colored pigment production in culture amended with Mn sulfate, but not control. Assay is scored as positive for Mn oxidation (1).

Conclusions

1. Two *Phaeoacremonium* spp. oxidize manganese *in vitro*. *Phaeoacremonium angustius* s.l. and *P. viticola* s.s. oxidize Mn. *Phaeoacremonium aleophilum* had no visible physiological response to manganese sulfate-amended media. Remaining *Phaeoacremonium* spp. were negative or resutls inconclusive (Table 1).

2. The genebank sequence AF197974 from the *ex-type* culture CBS 249.95 deposited by Groenewald et al. (2001) matches ITS rDNA sequences from isolates obtained during our New York and Pennsylvania survey (See isolate 316 for example). However, the *ex-type* culture of *P. angustius* is *P. aleophilum* based on ITS rDNA re-sequencing and physiological response to manganese sulfate-amended media (Table 1, Fig 5). Based on ITS rDNA sequences, *P. angustius* s.l. (AF197974, 316 and others included here) and *P. viticola* s.s. are not identical.

3. Isolates of *P. mortoniae* had a variable response to Mn sulfate-amended media. Crystals were never observed in Mn sulfate-amended media, but a brown rust colored pigment was observed near the hyphal tips. The *ex-type* culture of *P. mortoniae* produced some rust colored pigment near the hyphal tips, but it was not as strong as in isolates obtained from our survey and scored as a negative.

4. *Phaeomoniella chlamydospora* (Pch) exhibited reduced growth at 3000 ppm manganese-sulfate and did not grow at higher concentrations, nor was there a response indicating the presence of oxidize manganese.

5. *Stereum hirsutum* and *Phaeacremonium* spp. were manganese sulfate-tolerant with no reduced growth even at the highest concentration of Mn sulfate (6000 PPM).

6. *Fomitiporia punctata* was Mn sulfate sensitive exhibiting reduced growth at 300 PPM and no growth at higher concentrations. Both the PDA control (no Mn sulfate) and Mn sulfate-amended plates had notable pigment production and a qualitative determination of Mn oxidation could not be determined.