Phytopathol. Mediterr. (2006) 45, S131–S134

Manganese oxidation in Petri disease fungi as a novel taxonomic character

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Summary. Manganese (Mn) oxidation was evaluated for Petri disease fungi by using potato dextrose agar (PDA) amended with Mn sulfate. *Phaeomoniella chlamydospora* showed reduced growth on PDA amended with 3000 ppm Mn sulfate and exhibited no growth at higher concentrations. All *Phaeoacremonium* spp. evaluated were manganese tolerant and grew on PDA amended with as much as 6000 ppm Mn sulfate. *Phaeoacremonium viticola* and *P. angustius* s.s. oxidized manganese *in vitro*. *Stereum hirsutum* and *Fomitiporia punctata*, known white-rot basidiomycetes, were used as controls. *Stereum hirsutum* oxidized Mn *in vitro* and grew on PDA amended with 6000 ppm Mn sulfate. *Fomitiporia punctata* had reduced growth at 300 ppm and no growth at higher concentrations of Mn sulfate with no clear evidence of Mn oxidation.

Key words: lignin degradation, Mn-dependent peroxidase, esca, white-rot fungi.

Introduction

Phaeoacremonium W. Gams, Crous & M.J. Wingfield species, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & L. Mugnai) Crous & W.Gams (Crous & Gams, 2000) and white-rot fungi are associated with a decline of mature grapevines (Mugnai *et al.*, 1996; Larignon and Dubos, 1997; Mugnai *et al.*, 1999) called esca, while *P. chlamydospora* and *P. aleophilum* are associated with Petri disease, a decline affecting young vines. *Phaeomoniella chlamydospora* has a number of morphologically distinct characters that make it easy to identify. Identifying species within *Phaeoacremonium* based on morphology can be difficult, which led the authors to consider other possible characters. Mugnai *et al.* (1999) showed that *P. chlamydospora* and *P. aleophilum* produced trace amounts of ligninolytic enzymes in the peroxidase family. Mugnai *et al.* (1999) reported that isolates of the white-rot basidiomycetes *Fomitiporia punctata* (Fr.) Murrill (= *F. mediterranea* M. Fischer) and *Stereum hirsutum* (Willd. : Fr.) S. F. Gray could be isolated from esca-diseased grapevines at different stages of wood decay. Fischer (2002) showed that European isolates, included under the name *F. punctata* from grape, were a distinct species, *F. mediterranea*.

White-rot fungi produce four families of oxidative enzymes involved in lignin degradation. They are: lignin peroxidase (LiP), laccase, manganese-independent peroxidase (MIP), and manganese-dependent peroxidase (MnP) (Moreira *et al.*, 1999). MnP oxidizes Mn (II) to Mn (III), which then acts as a diffusible agent that can oxidize lignin moieties (Glenn and Gold, 1985). Manganese sulfate-

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amended agar plates can be used to assay for manganese-dependent peroxidase because oxidized manganese will form a brown pigment or black crystals of Mn dioxide *in vitro* (Glenn and Gold, 1985; Otrosina and Illman, 1994).

The objectives of this study were to determine the ability of *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. to oxidize Mn *in vitro* and to assess its taxonomic usefulness. In addition, understanding of the type of peroxidase enzyme produced by *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. could provide insight into the ecological role these fungi play in Petri disease and esca.

Materials and methods

Identification of fungal strains

The ITS rDNA region was re-sequenced from the following '*ex-type*' cultures of *Phaeoacremonium*: *P. angustius*, CBS 249.95 contaminated by *P. aleophilum* (Mostert *et al*., 2005); *P. rubrigenum* W. Gams, Crous & M.J. Wingfield, CBS 498.94; *P. aleophilum*, CBS 246.91; *P. viticola* J. Dupont, CBS 101738; *P. inflatipes* W. Gams, Crous & M.J. Wingfield, CBS 391.71; *P. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingfield, CBS 860.73; *P. mortoniae* Crous & W. Gams, CBS 101585. Additional isolates were obtained from a vine decline survey of the Northeast USA from Pennsylvania (PA) and New York (NY) (Stewart and Wenner, 2004) and identified to species using standard morphology and ITS rDNA sequences. Isolates are numbered in the order they were established in pure culture from the survey with the collector's initials, Elwin L. Stewart (ELS). The isolates used and species identifications follow: *P. angustius* s.s., ELS316; *P. rubrigenum*, ELS322; *P. aleophilum*, ELS287; *P. mortoniae* s.l., ELS170, ELS285; *Phaeomoniella chlamydospora*, ELS178. ITS rDNA sequence data was generated following the protocols outlined in Stewart *et al*. (1999) and Chaverri *et al*. (2001) and can be provided upon request. Isolates of *Stereum hirsutum* (HHB-4401-Sp) and *Fomitiporia punctata* (FP-133888-3p) from North America were obtained from the culture collection at the Forest Products Laboratory (FPL), Madison Wisconsin. The ITS rDNA region was not sequenced from the basidiomycetes used in this study.

Manganese assay

Isolates were grown on Difco potato dextrose agar (PDA) amended with the following concentrations of Mn sulfate, FW 151 (Sigma, Cat. # M-7634, St. Louis, MO, USA): Control (no Mn sulfate); 22 ppm (22 mg $MnSO_4\,l^{-1}$); 80 ppm (80 mg $MnSO_4\,l^{-1}$); 100 ppm (100 mg $MnSO_4\,l^{-1}$); 300 ppm (300 mg $MnSO_4\,l^{-1}$); 1000 ppm (1 g $MnSO_4\,l^{-1}$); 2000 ppm (2 g $MnSO_4\,l^{-1}$); 3000 ppm (3 g $MnSO_4\,l^{-1}$); 4000 ppm (4 g $MnSO_4\,l^{-1}$); 5000 ppm (5 g $MnSO_4\,l^{-1}$); 6000 ppm (6 g $MnSO_4\,l^{-1}$). Cultures were grown at room temperature for 20 d, alternating 12 hours in darkness, and 12 hours under cool fluorescent light. The assay was replicated twice. Positive results were recorded when isolates produced black crystals on $MnSO_4$ amended media or when isolates turned $MnSO_4$ amended media a rust color outside of the area delimited by hyphal growth. Intermediate reaction types and reduction in fungal growth on the $MnSO_4$ amended media were also noted.

Results and discussion

Phaeomoniella chlamydospora did not oxidize Mn *in vitro* and showed reduced growth (RG) at 3000 and 4000 ppm Mn sulfate and no growth (NG) at higher concentrations. The *Fomitiporia punctata* isolate showed RG at 300 ppm Mn sulfate and NG at higher concentrations. *Fomitiporia punctata* produced such copious amounts of a dark brown pigment on both the control and Mn sulfate-amended plates that it was not possible to observe Mn oxidation. *Stereum hirsutum* and all *Phaeoacremonium* species tested (Table 1) were Mn-tolerant and grew well at all concentrations of Mn used in this study. *Stereum hirsutum*, *P. viticola* and *P. angustius* s.s (ELS316) oxidized Mn *in vitro* (Table 1). *Stereum hirsutum* formed a brown pigment on all plates amended with Mn sulfate. *Phaeoacremonium viticola* and *P. angustius* s.s. produced black crystals putatively of Mn dioxide on plates amended with Mn sulfate 20–30 days after inoculation. The ITS rDNA sequence of isolate ELS316 was identical to the original sequence deposited by Groenewald *et al*. (2001) in GenBank for *P. angustius*. Crystal formation for *P. viticola* and *P. angustius* was reduced on agar plates amended with the 22 ppm, 80 ppm, and 100 ppm Mn sulfate. The '*ex-type*' culture of *P. aleophilum* did not oxidize Mn *in vitro*. The '*ex-type*' culture of *P. angustius* CBS

Table 1. Manganese oxidation in esca fungi. Formation of brown pigment or black crystals indicates positive reaction or oxidation of Mn (II) to Mn (III) (1), negative reactions are scored as (0), uncertain reactions (0/1); reduced growth (RG); no growth (NG).

Fungal species	Mn sulphate concentration (ppm)										
	Control (no Mn)	22	80	100	300	1000	2000	3000	4000	5000	6000
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	NG	NG	NG	NG	NG
Phaeoacremonium angustius CBS 249.95	0	0	0	0	0	0	0	0	0	0	0
Phaeoacremonium angustius ELS316	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 ELS322	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 ELS287	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 ELS170	0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Phaeoacremonium mortoniae ELS285	0	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Phaeomoniella chlamydospora ELS178	0	0	0	0	0	0	0	RG	RG	NG	NG

249.95 did not oxidize Mn and re-sequencing of the ITS rDNA from this '*ex-type*' produced a sequence that matched the '*ex-type*' of *P. aleophilum*, which is consistent with findings of other researchers that the '*ex-type*' of *P. angustius*, CBS 249.95, has been contaminated over time with *P. aleophilum*. Therefore, isolate ELS316 is *P. angustius* s.s and manganese oxidation can be used to differentiate this species from *P. aleophilum*. The '*ex-type*' culture of *P. mortoniae* did not oxidize Mn, but isolates ELS170 and ELS285 (*P. mortoniae* s.l., 3 base pairs different from the '*ex-type*'), obtained from the survey, had an intermediate reaction on plates amended with Mn sulfate, producing a brown pigment near the hyphal tips. It is possible that isolates ELS170 and ELS285 (*P. mortoniae* s.l.) represent an undescribed species given the variation in the ITS rDNA region (Stewart, unpublished) and their response to Mn sulfate-amended media. All other *Phaeoacremonium* species sampled were negative for Mn oxidation. Photographs showing the reactions on manganese can be found at http://grape.cas.psu.edu. The differential responses of *Phaeomoniella* (Mn-intolerant) and *Phaeoacremonium* (Mn-tolerant), as well as the differential response within the genus *Phaeoacremonium* (oxidizing species vs. non-oxidizing species) suggests that Mn sulfate-amended media could be used as a taxonomic character, or an initial screen for grouping isolates as they are obtained from the field. Black oxidized Mn can easily be visualized in the oxidizing *Phaeoacremonium* species at 20 days on 3000 ppm Mn sulfate-amended media, and growth on PDA amended with 3000 ppm Mn sulfate can easily distinguish isolates of *Phaeomoniella* (reduced growth) from *Phaeoacremonium*. However, many more isolates from different geographical locations must be sampled before establishing this assay as a standard practice in taxonomic studies.

Mugnai *et al*. (1999) suggested that Petri disease fungi are thought to prepare the wood for colonization by *Fomitiporia punctata* (= *F. mediterranea*). Based on our results, two species of *Phaeoacremonium* spp., *P. viticola* and *P. angustius* s.s. have a Mn-dependent peroxidase or MnP-like peroxidase while the other species tested in this genus are Mn- tolerant. *Phaeomoniella chlamydospora* did not oxidize Mn *in vitro* and was relatively Mn-intolerant, suggesting a lack of MnP. *Stereum hirsutum* has a well-characterized Mn-dependent peroxidase involved in lignin degradation (Morei-

ra *et al*., 1999). Based on this assay, *S. hirsutum* is Mn-tolerant, and able to oxidize Mn *in vitro*. *Fomitiporia punctata* was also Mn-intolerant. It is possible that *F. mediterranea* responds differently to manganese than *F. punctata* and manganese assays using strains of *F. mediterranea* from Europe should be evaluated. Collectively, these observations raise questions regarding the ecological interaction between the fungi involved in the esca disease complex, especially with regard to the mode of lignin degradation.

Acknowledgements

This research was supported by grant funds from the Pennsylvania Department of Agriculture (ME# 443256), the Pennsylvania Wine Marketing and Research Board (ME# 443448, ME# 444468), and the Viticulture Consortium East. We are grateful to Dr. Harold Burdsall Jr. for the cultures of *Stereum hirsutum* and *Fomitiporia punctata*.

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Accepted for publication: January 24, 2006