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Recovery of *Phaeomoniella chlamydospora* and *Phaeoacremonium inflatipes* from soil and grapevine tissues

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Summary: Rose Bengal Chloramphenicol Agar (RBCA) appears to be a suitable media for isolating *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* from soil, spore traps and plant tissue. Using the soil-plate method, populations of these organisms were recovered from the soil and surfaces of plant tissue from many different regions of California. In addition, in a few vineyard sites these fungi were recovered from dried plant sap, which had oozed from grapevine girdling wounds and from standing water under grapevine drip systems. RBCA, along with a filtering system, is a useful tool in determining the presence of Petri disease pathogens in vineyard soils, water, and plant tissues. This research presents the first report of the recovery of *Phaeoacremonium inflatipes* from soil and standing water under grapevines.

Key words: selective medium, RBCA, Petri disease, mitosporic fungi.

Introduction

Fungi associated with Petri disease[1] in California include *Phaeomoniella chlamydospora* (Crous *et al*., 2000), (formerly *Phaeoacremonium chlamydosporum*), *Phaeoacremonium inflatipes,* and *Phaeoacremonium aleophilum* (Crous *et al*., 1996). These fungi have been documented in all major grape-growing regions in California and pathogenicity tests have confirmed they are effective pathogens (Scheck *et al*., 1998b; Khan *et al*., 1999). Work by Khan *et al*. (1999) demonstrated that *Phaeoacremonium* spp. are root pathogens, while *Pa. chlamydospora* is more a wound pathogen. Recent studies have also shown that spores of these fungi are present in vineyards, as aerial spore traps have confirmed their presence (Larignon *et al*., 2000; Eskalen *et al*., 2001). However, the presence of these fungi in the soil has never been documented.

Due to their slow growth on standard media, *Phaeoacremonium* spp. and *Pa. chlamydospora* are often difficult to recover from infected wood, spore traps, and soil. Moreover, contamination from faster growing fungi and bacteria often inhibits the ability of *Phaeoacremonium* spp. and *Pa. chlamydospora* to grow. Rose Bengal Chloramphenicol Agar (RBCA) is a medium often used for enumerating yeast and molds in food for product evaluation (Jarvis, 1973). Rose bengal has also been used as a selective agent for isolating yeast and fungi

[1] At the general Assembly of the 2nd ICGTD meeting held in Lisbon 2001 it was unanimously decided that young grapevine decline, 'black goo', Petri vine decline will henceforth be called Petri disease.

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from the soil (Martin *et al*., 1951; Miller *et al*., 1954). The pH, rose bengal and added chloramphenicol tend to suppress the growth of most bacteria (Smith *et al*., 1944). Additionally, rose bengal when taken up intracellularly by most fungi tends to limit their size and growth rate. This can prevent the overgrowth of slow growing fungi by faster growing species. The purpose of this research was to test a semi-selective medium that would minimize contaminants while allowing growth of *Pa. chlamydospora* and *Phaeoacremonium* spp., and to use this medium to determine if these fungi naturally occur in vineyard soils and grapevine tissues.

Materials and methods

Soil samples.

Soil samples were collected from eleven grape-growing counties in California (Fig. 1). Samples were taken from vines showing esca and Petri disease symptoms. In addition, a few soil samples were taken from underneath healthy vines.

Grapevine girdling-wound sap

At the time soil samples were taken, many of the table grape vineyards being examined had recently been girdled. During the spring, girdling is performed on the vines of many table grape varieties in California to increase the size of the berries. Using a girdling knife a wound is made completely around the trunk, removing the outer bark and exposing the vascular cambium. This prevents photosynthates from traveling down the vine and enlarges the fruit beyond normal size. This practice creates a larger, more appealing and marketable grape for the consumer. Because girdling had recently occurred in vineyards that were sampled, wounds on grapevine trunks were still oozing with xylem sap. This sap was collected for analyses as well.

Grapevine bark and standing water

Old bark shedding from vines showing esca and Petri disease symptoms was also collected. In a few vineyards with approximately 50% of the vines showing esca symptoms, water was collected from underneath the vines. The standing water was collecting near the base of grapevines and directly underneath irrigation drip line emitters. Fruit mummies and other grapevine debris could be found in and around these water puddles. Water samples were also taken directly from the irrigation drip line before reaching the ground. In total 73 samples were collected (Table 1).

Using the soil plate method (Dhingra *et al*., 1995), soil and bark samples were placed in sterile water and diluted to 10^{-3} concentration. Girdling-wound sap samples were washed in sterile water and diluted to 10^{-1}. Water samples were maintained at full strength. The appropriately diluted solutions from soil, bark and sap washings as well as water samples were then loaded into Monoject 35 cc sterile syringes. Attached to each syringe was a 5 μm filter followed by a 0.45 μm filter. Upon passing through the syringes, larger spores and dirt particles from the suspensions were trapped on the 5 μm filters, while smaller spores, including those of *Phaeoacremonium* spp. and *Pa. chlamydospora,* were trapped on the 0.45 μm filters. The 0.45 μm filters were then removed, flipped upside down and placed in sterile microfuge tubes. Aliquots of 1 ml sterile water were backwashed through the filters to dislodge any spores trapped on the filters. These suspensions were then aliquoted out in 200 μl samples onto plates of RBCA [Mikrobiologie Co.; distributed by EM Science, Gibbstown, NJ, USA; composition (g l^{-1}): mycological peptone: 5.0, glucose: 10.0, di-potassium hydrogen phosphate 1.0, magnesium sulfate 0.5, rose bengal 0.05, chloramphenicol 0.1, agar agar 15.5] and spread using an L-rod. Plates were left cracked open in a flow hood for 15 minutes to allow suspensions on the agar surface to dry. Plates were then stored at room temperature for approximately 10–14 days to allow growth of fungi. As stated before, RBCA limits many fungi and bacteria while allowing *Phaeoacremonium* spp. and *Pa. chlamydospora* to grow. However, other fungi with spores of a similar size caught by the 0.45 μm filters also grow on RBCA. After the 10–14 days, colonies were examined and possible *Phaeoacremonium* spp. or *Pa. chlamydospora* colonies were subcultured onto PDA amended with 0.1 g tetracycline l^{-1} (PDA-tet). PDA-tet plates were allowed to grow for another 10–14 days and then examined for any cultures of *Phaeoacremonium* spp. and *Pa. chlamydospora*.

Results and discussion

A map of the state of California showing the eleven counties that were surveyed is seen in Fig-

Fig. 1. Map of California showing the eleven viticulture counties surveyed (map modified and used with permission from the U.S. Census Bureau).

Table 1. Summary of vineyard samples collected to test for the presence of *Phaeacremonium* spp. and *Phaeomoniella chlamydospora*.

Sampling date	Site	Location (County)	Symptoms	Sample type	No. of samples	Results
5/8/01	Car	Kern	Esca	Soil	2	(-)[a]
6/20/01	Car	Kern	Esca	Soil	1	(-)
6/20/01	Car	Kern	Esca	Puddle of water under vine	4	(+) *Pm. inflatipes*
6/20/01	Car	Kern	Esca	Girdling wound sap	2	*(+) Pa. chlamydospora*
6/20/01	Car	Kern	Esca	Water from irrigation tube	2	(-)
7/18/01	Car	Kern	Esca	Soil	2	(-)
7/18/01	Car	Kern	Esca	Puddle of water under vine	1	(+) *Pm. inflatipes*
7/18/01	Car	Kern	None	Puddle of water under vine	1	(-)
7/18/01	Car	Kern	Esca	Water from irrigation tube	2	(-)
7/18/01	Car	Kern	Esca	Bark at soil level	1	(-)
7/18/01	Car	Kern	None	Bark at soil level	1	(-)
7/18/01	Car	Kern	Esca	Girdling wound sap	1	(-)
7/18/01	Car	Kern	Esca	Girdling wound sap	1	(-)
5/8/01	Delano	Kern	Esca	Soil	2	(-)
6/8/01	Diablo	Contra Costa	Declining	Soil	2	(+) *Pm. inflatipes*
6/8/01	Rose	Contra Costa	Only virus symptoms	Soil	2	(+) *Pm. inflatipes*
6/8/01	Kess	Contra Costa	Esca	Soil	1	(-)
6/8/01	5S	Contra Costa	None	Soil	1	(-)
6/8/01	Bloom	Contra Costa	Declining	Soil	1	(-)
6/20/01	Fire	San Luis Obispo	Esca	Soil	3	(-)
6/20/01	Shan	San Luis Obispo	Esca	Soil	1	(-)
6/20/01	John	Madera	Esca	Soil	2	(-)
7/18/01	John	Madera	Esca	Soil	1	(-)
7/18/01	John	Madera	Esca	Girdling wound sap	4	*(+) Pa. chlamydospora*
7/18/01	John	Madera	Esca	Bark at soil level	2	*(+) Pa. chlamydospora*
6/29/01	Herz	Sacramento	None	Soil	2	(-)
7/11/01	Soda	Napa	Declining	Soil	2	(-)
7/18/01	Lodi	San Joaquin	Esca previous year	Soil	1	(-)
5/8/01	Newt	Santa Barbara	Declining	Soil	2	(-)
5/8/01	Gainey	Santa Barbara	Declining	Soil	2	(-)
6/20/01	Tulare	Tulare	Esca	Girdling wound sap	2	*(+) Pa. chlamydospora*
7/18/01	Goed	Tulare	Esca	Soil	1	(-)
7/18/01	Goed	Tulare	Esca	Girdling wound sap	1	(-)
7/18/01	Goed	Tulare	None	Girdling wound sap	2	(-)
7/18/01	Goed	Tulare	Esca	Bark at soil level	2	(-)
8/8/01	Mad	El Dorado	Esca (5 varieties)	Soil	5	(-)
8/8/01	Bogg	El Dorado	Esca (3 varieties)	Soil	3	(-)
8/10/01	Sebas	Sonoma	Declining	Soil	2	(+) *Pm. inflatipes*
8/10/01	Sebas	Sonoma	Declining	Puddle of water under vine	3	(-)
Total No.	20	11	34		73	9
Pa. chlamydospora		3 (27%)[b]			4 (5%)[b]	
Pm. inflatipes		3 (27%)[b]			4 (5%)[b]	

[a] (-), no pathogens recovered.
[b] Percentage over total No. of Counties surveyed or of samples collected.

ure 1. Table 1 contains results obtained from sampling done throughout the state. Of the *Phaeoacremonium* spp. associated with grapevines, only *Pm. inflatipes* was isolated from soil or standing water. Populations of *Pm. aleophilum* were not recovered from any samples. Of the eleven vineyard counties in which soils were tested, four samples in three (27%) different counties tested positive for *Pm. inflatipes*. One of these soils was from a Contra Costa County vineyard in which vines showed severe stunting and decline symptoms. Previous isolations from vines in this vineyard had shown both *Phaeoacremonium* spp. and *Pa. chlamydospora* to be present. The other location was also in Contra Costa County and also a vineyard showing overall stunted growth. However, the only pathogens that had been previously recovered from these vines were viral. Therefore two sites, one with a history of *Phaeoacremonium* spp. and *Pa. chlamydospora*, the other without, both contained populations of *Pm. inflatipes* in the soil. *Pm inflatipes* was also recovered from a vineyard in Sonoma County with declining vines, known to have a history of *Pa. chlamydospora* and *Pm. inflatipes*.

Surprisingly, the standing water samples taken from underneath symptomatic esca vines in Kern County also had populations of *Pm. inflatipes* recovered from them. Water taken from irrigation tubes before reaching the ground tested negative for *Pm. inflatipes*, indicating this inoculum probably came from the soil or grapevine debris rather than from the water source. Different puddles were tested during separate visits to the vineyard, with positive results. As mentioned previously, many puddles contained old fruit mummies and grapevine debris. Perhaps inoculum of *Pm. inflatipes* was overseasoning and surviving on this plant debris.

Pa. chlamydospora was not recovered from any of the soils tested, but was present both in plant sap and on the outer bark of grapevines. It is still not known whether this fungus is incapable of surviving in the soil, or whether we were just unable to recover it. Of the eleven counties, three (27%) had *Pa. chlamydospora* recovered from either old bark or plant sap. In Kern County and Tulare County vineyards, sap taken from vine girdling-wounds contained populations of *Pa. chlamydospora*. It is not known whether fungal spores merely landed on the wounds and colonized them in this manner, or whether these vines were already infected with *Pa. chlamydospora* and oozed infected sap when wounded. Previous research in our lab has shown that these fungi can be isolated from the sap bleeding from pruning wounds in the early springtime before bud burst occurs (Gubler, unpublished). This may be the explanation for the recovery of *Pa. chlamydospora* from the sap. However, if infection occurred from aerially disseminated spores landing on girdling wounds, this may be an important means of infection. In a Madera county vineyard, bark tissue sloughing from symptomatic vines was collected and analyzed as well. Fungal populations of *Pa. chlamydospora* were recovered from this tissue, indicating this as a possible site for overseasoning of *Pa. chlamydospora*. It is possible that old bark sloughing from infected grapevines contains mycelium, or more likely pycnidia, of *Pa. chlamydospora* and is acting as an inoculum source. Previous reports have shown that pycnidia of *Pa. chlamydospora* do in fact survive on grapevine bark tissue (Edwards *et al*., 2001a, 2001b). Perhaps pycnidia were present in the bark pieces that were sampled, and just not identified at the time in our study.

This work further documents the importance of *Pm. inflatipes* as a soilborne pathogen in California vineyards. This is the first reported successful recovery of *Pm. inflatipes* from soil and from standing water in vineyards. This research also documents *Pa. chlamydospora* on the bark surface of grapevines for the first time in California vineyards. The presence of *Pa. chlamydospora* in sap exuding from girdling wounds also raises some serious questions about the epidemiology of this fungus. In addition this research introduces the use of a selective medium, RBCA, which is capable of recovering *Phaeoacremonium* spp and *Pa. chlamydospora* from the soil, bark, water and girdling-wound sap. RBCA, along with the filtering method described is a reliable assay for testing the presence of these fungi from various substrates. More extensive statewide sampling using this method is needed for a complete understanding of these pathogens and their means of survival in vineyards.

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