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Pathogenicity of *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* on grape berries in California

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Summary. Injured and non-injured grape berries were inoculated with spore suspension of *Phaeomoniella chlamydospora* or *Phaeoacremonium aleophilum* under field (intact berries) and laboratory (detached berries) conditions. In one test, berries were injured by pricking the skin with a syringe needle to a depth of approximately 1.5 mm. Brown to purple lesions appeared 5 to 7 days after inoculation in both the injured intact and detached berries. Lesions on these berries were larger when inoculated earlier in the season indicating that young, immature berries are more susceptible to infection than mature berries. In another test, berries were injured by rubbing the skin with carborundum dust using a cotton-tipped applicator. Esca-like lesions developed in 4 to 5 days after inoculation of detached but not intact berries. Occasional infection of non-injured berries occurred which appeared as small dots to pin-head size lesions around the lenticels. Scanning electron microscopy observations of these lesions showed abundant hyphal growth on the surface with apparent penetration through lenticels; however, fungal structures were not detected with certainty beneath the lenticels or intact cuticle. In both tests, the fungi were re-isolated from the advancing margin of the lesions.

Key words: *Vitis vinifera*, esca, Petri disease.

Introduction

Esca is one of the most important diseases of grapevines worldwide. In California, the incidence of esca has continuously increased over the last 10 years especially on young grapevines (2–6 years). The most prominent symptoms of the disease are spotting of berries and interveinal and marginal leaf necrosis that develops into

a tiger-striped pattern (Mugnai *et al.*, 1999). Symptoms of esca on berries have been reproduced by inoculation of pruning wounds of spurs, branches, or trunks of standing vines (Sparapano *et al.*, 2001; Feliciano *et al.*, 2004). However, no study has been done on reproduction of esca symptoms by inoculation of grape berries. Three organisms have been consistently associated with esca in California vineyards: *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, and *Phaeoacremonium inflatipes*¹ (Scheck *et al.*, 1998; Eskalen and Gubler, 2001). The present study was conducted to determine the pathogenicity of *Pa. chlamydospora* and *Pm. aleophilum* on injured and non-injured grape berries and study the histology of infection.

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¹ Isolates that were previously identified as *Phaeoacremonium inflatipes* are now classified as *Phaeoacremonium aleophilum*, based on results of recent morphological and molecular tests (Rooney-Latham *et al.*, 2004).

Materials and methods

Pathogenicity tests were conducted under field and laboratory conditions using intact and detached berries, respectively. Injured and non-injured berries were inoculated with spore suspension (approximately 1×10^6 spores ml^{-1} of water) of *Pa. chlamydospora* strain P99.16, *Pm. aleophilum* strain P99.10, or *Pm. aleophilum* strain P99.4 (previously identified as *Phaeoacremonium inflatipes*), isolated from grapevines diagnosed with Petri disease in California. Control berries were inoculated with sterile distilled water. In one test, berries were injured by pricking the epidermis with a syringe needle. In another test, berries were injured by lightly rubbing the epidermis with carborundum dust.

Inoculation of berries injured by pricking

Field inoculations

Two vines and 4 clusters per vine (2 injured, 2 uninjured) of cv. Thompson Seedless and Carignane were inoculated at pea-size or 2 weeks after pea-size. For each cultivar, treatments were arranged in a completely randomized design. At least 15 berries were inoculated per cluster; of these, 5 berries were marked for disease assessment. Berries were pricked with a syringe needle to a depth of approximately 1.5 mm and immediately inoculated by misting the inoculation sites with the spore suspension or water using an atomizer. Inoculated clusters were covered overnight with plastic bags. Berries were observed for lesion development and lesion size was recorded 15 days after inoculation.

Laboratory inoculations

Detached berries of cv. Thompson Seedless, Cabernet Sauvignon, Chardonnay, and Carignane were surface sterilized in 0.5% NaOCl for 3 min, rinsed twice with sterile distilled water, and air-dried. Berries were inoculated on the first week of June and at weekly intervals thereafter for 6 weeks. At the first inoculation date, Thompson Seedless was at pea-size stage while Cabernet Sauvignon, Chardonnay, and Carignane were approximately 3, 6, and 2 days after pea-size, respectively. Berries were injured as above and immediately inoculated by depositing 20 μl of inoculum onto each inoculation site using a micropipette. For each cultivar, there were 4 replications with 5 berries each, arranged in a completely randomized design

inside plastic crispers lined with wet towel papers. Inoculated berries were incubated at room temperature ($24 \pm 2^\circ\text{C}$) and observed for lesion development. Lesion size was measured 7 and 14 days after inoculation. At each inoculation date, soluble solids and pH of the berries were recorded.

Inoculation of berries injured by carborundum

In field and laboratory tests, berries of cv. Thompson Seedless and Chardonnay were inoculated at pea-size and 2 weeks after pea-size stage. Berries were injured by lightly rubbing the skin with carborundum dust using a cotton-tipped applicator and immediately inoculated by touching the injured area with a cotton-tipped applicator that had been dipped in the spore suspension. The number of samples inoculated and incubation conditions were the same as described above. Observations were made daily on symptom appearance.

Reisolation of the pathogen

Sections from the leading edge of the lesions were surface-sterilized for 1 min in 0.5% NaOCl, rinsed twice with sterile distilled water, and plated in Potato-dextrose agar amended with tetracycline (PDA-tet). The plates were incubated at room temperature and observed for fungal growth after 7 days.

Histological studies

Samples of inoculated injured and non-injured berries of cv. Chardonnay were cut in small pieces and stored in formalin-alcohol-acetic acid (FAA) fixative for histological examination. Samples were processed for light and scanning electron microscopy observations as described previously (Feliciano and Gubler, 2001).

Results

Lesion development in berries injured by pricking

In the field test, lesions developed on Thompson Seedless and Carignane berries 5 to 7 days after inoculation. The lesions were purplish on berries inoculated with *Pa. chlamydospora* and tan to brown in berries inoculated with *Pm. aleophilum*, sometimes with profuse sporulation. In Thompson Seedless, lesion size ranged from 2 to 3 mm and did not differ significantly ($P > 0.05$) between organisms or date of inoculation. In Carignane, lesion size did not differ significantly ($P > 0.05$) between

dates of inoculation but lesions were significantly smaller ($P < 0.05$) in berries inoculated with *Pa. chlamydospora* (1.8 to 2 mm) than in those inoculated with the two *Pm. aleophilum* isolates (2.7 to 4.2 mm). Light microscopy observations of paraffin sections of lesions showed extensive periderm formation in berries inoculated with the fungi (Fig. 1A and B). Control berries did not develop lesions but also formed periderm in response to wounding (Fig. 2A and B). In uninjured berries inoculated with the pathogen, small purplish dots, which developed to pin-head size after 2 weeks, were sometimes observed around the lenticels. Scanning electron microscopy observations of these berries showed abundant hyphal growth on the surface with apparent penetration through the lenticels (Fig. 3); however, fungal structures were not de-

tected with certainty beneath the lenticels or intact cuticle.

In the laboratory test, lesion development followed the same general pattern in all the cultivars. Lesion size decreased as the berries matured; however, differences were not always significant. Lesion size did not differ significantly ($P > 0.05$) among the fungal isolates except in Thompson Seedless where lesions were significantly smaller ($P < 0.05$) in berries inoculated with *Pm. aleophilum* strain P99.4. Among the cultivars, lesions were larger in Chardonnay. Based on the average of all fungal isolates, lesion size in this cultivar ranged from 6.1 to 6.5 mm at the earlier inoculation dates (June 6–18) and from 0 to 3.1 mm at the later inoculation dates (June 27–July 23). No lesions developed in all the cultivars at the last inoculation date (July



Fig. 1. A. Lesion (arrow) produced on grape cv. Chardonnay 10 days after pricking and inoculation with *Pm. aleophilum* P99.4, 10 \times . B. Paraffin section of the wound area showing pronounced periderm formation (arrow) (400 \times).

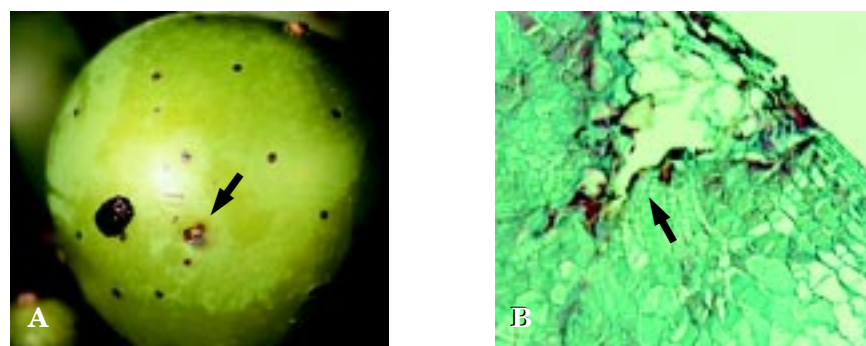


Fig. 2. A. Wound (arrow) produced on grape cv. Chardonnay 10 days after pricking and inoculation with distilled water, 10 \times . B. Paraffin section of wound area showing periderm formation (arrow) (400 \times).

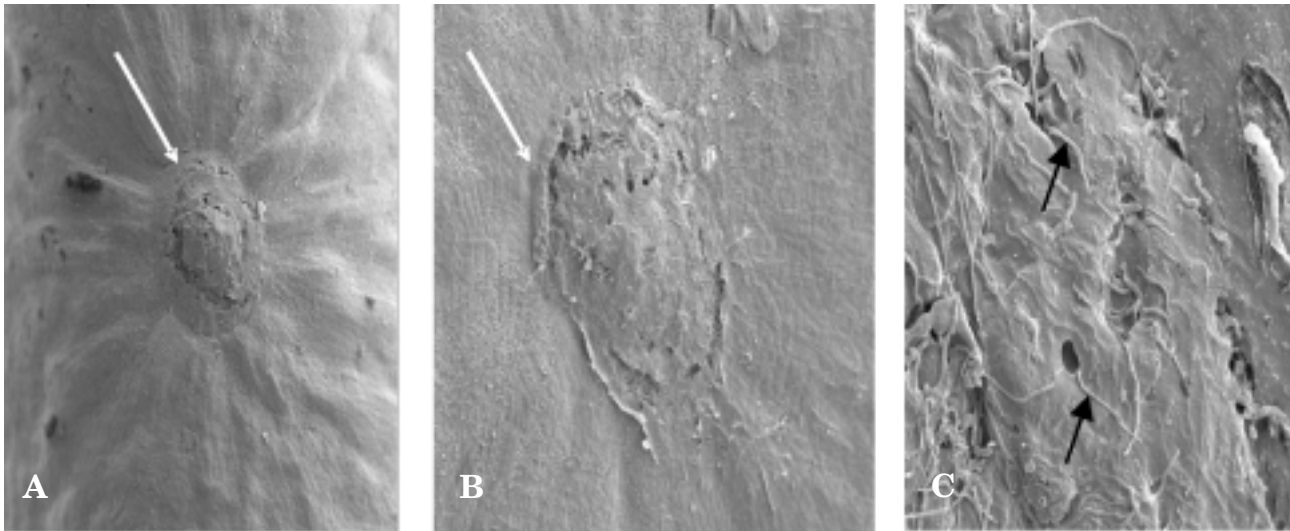


Fig. 3. Scanning electron micrographs of the surface of grape berries cv. Chardonnay showing the lenticel area (white arrows). A. Control. B. Inoculated with *Pm. aleophilum* P99.4, 72 h after inoculation. C. Enlarged portion of the inoculated lenticel showing hyphae entering cracks in the lenticel (black arrows).

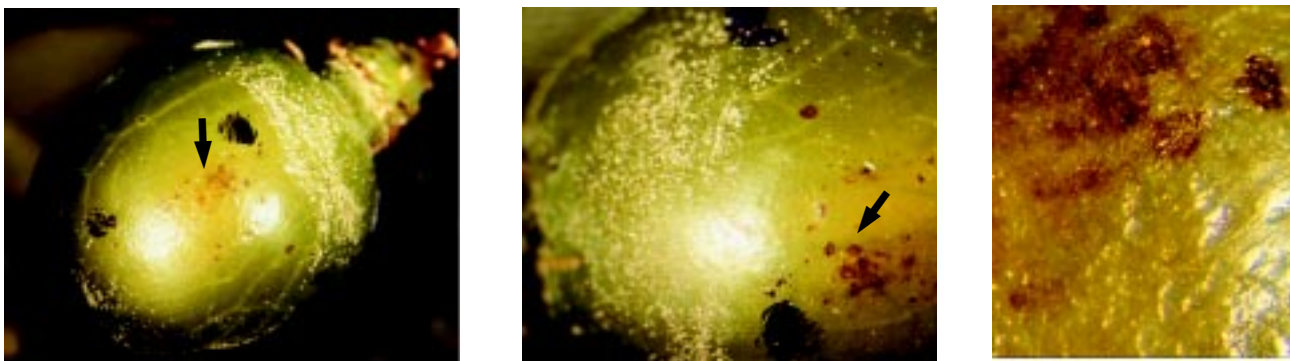


Fig. 4. Esca-like lesions (arrows) produced when berries were rubbed with carborundum dust prior to inoculation with *Pm. aleophilum* P99.4. Photos from left to right show increasing magnification, 6.3, 10, 20 \times . Similar lesions were produced on berries inoculated with *Pm. aleophilum* P99.10 and *Pa. chlamydospora* P99.16.

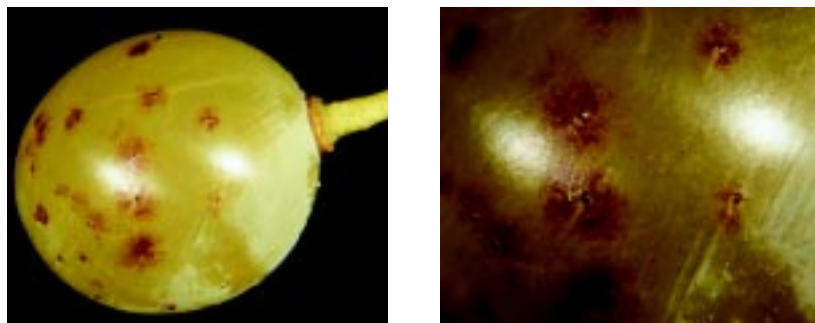


Fig. 5. Typical symptom of esca on grape berry with epidermal wax removed. Photos show increasing magnification from 8 to 20 \times .

23). Total soluble solids increased as the berries matured, from 3.9 (June 6) to 13.5 (July 23) in Chardonnay, 4.0 to 7.8 in Cabernet Sauvignon, 5.0 to 18.5 in Thompson Seedless, and 4.0 to 7.5 in Carignane; however, there were no consistent relationships between soluble solids and lesion size. No trends were observed in pH level as the berries matured.

Lesion development in berries injured by carborundum

No lesions developed in field inoculations of intact berries but lesions developed 4 to 5 days after inoculation of detached berries in the laboratory (Fig. 4). Lesions resembled naturally-occurring esca lesions (Fig. 5) and consisted of numerous brown to purple spots scattered over the carborundum-rubbed surface of the berries. The lesions initially appeared as minute spots that later enlarged and coalesced. The lesions appeared 1 to 2 days later and were slightly smaller on berries inoculated with *Pa. chlamydospora* than in berries inoculated with the 2 isolates of *Pm. aleophilum*. Light microscopy observation of paraffin sections of the lesions showed that hyphal growth inside the epidermal cell was slow, the hyphae was observed to penetrate only a few cells deep in the epidermal layer.

All the inoculated fungi were reisolated on PDA-tet from the advancing margin of the lesions after surface sterilization.

Discussion

Our results show that *Pa. chlamydospora* and *Pm. aleophilum* are pathogenic to grape berries. All the fungi were re-isolated from the advancing margin of the lesions that developed after inoculation of attached and detached berries injured by pricking with syringe needle and detached berries injured by rubbing the skin with carborundum dust. Inoculation of carborundum-injured berries produced esca-like lesions on detached but not in-

tact berries. The difference in results may be due to continuous availability of moisture for spore germination and mycelial growth in the laboratory test or the capacity of intact berries to heal superficial wounds rapidly. Symptoms of naturally-occurring esca are observed predominantly on the side of berries that are most exposed to external factors such as radiation and blowing dust or sand. The use of carborundum dust was meant to mimic injury caused by blowing dust or sand which sometimes will occur in California vineyards. Occasional infection of non-injured inoculated berries occurred but it is not clear whether the pathogen entered through the lenticels or through minute, undetectable breaks in the cuticle or minute injuries caused by insects or other agents. We were not able to demonstrate the presence of fungal structures beneath the lenticels or apparently intact cuticles.

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