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Histological investigations on infection of grape roots and shoots by *Phaeoacremonium* spp.

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Summary. Tissue-cultured grapevines cv. Cabernet Sauvignon were used to determine whether injury is a pre-requisite to penetration of shoot and root tissue by *Phaeoacremonium* spp. and to ascertain the path of invasion inside the host. Uninjured and injured roots and shoots were inoculated with *P. inflatipes* and *P. aleophilum* at approximately 10^6 spores ml^{-1}. Isolation and scanning electron microscopy observations showed that *Phaeoacremonium* spp. was capable of penetrating uninjured roots and shoots. Light microscopy observations of paraffin embedded sections showed that rapid spread of the fungus in roots was through the vascular tissues and intercellular spaces of the cortex. In the shoots, spread of the fungus was initially through the intercellular spaces of the epidermis, cortex, and pith; rapid spread apparently occurred in the intercellular spaces of the pith. Spores were seen in the pith area as well as in the xylem. Remote from the point of inoculation, hyphae were seen in the epidermis, cortex, pith, and vascular tissues without noticeable change in appearance of the invaded cells. Symptoms of the disease were observed in some plants 2 months after inoculation but isolations made in symptomless plants after 2 weeks showed the presence of the fungus in all parts of the plant.

Key words: *Phaeoacremonium*, Petri disease, young vine decline, histology.

Introduction

Petri disease (syn. young vine decline) is becoming an important problem of young grapevines in California vineyards. The three known causal agents of the disease in California are *Phaeoacremonium inflatipes*, *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* (Scheck *et al*., 1998). Infected grapevines show reduced trunk diameter, shortened internodes, small leaves, reduced amount of foliage, and brown to black spots or streaks in the xylem vessels of the rootstock (Adalat *et al*., 2000). It has been suggested that the organisms may exist as endophytes or as latent infections and may induce a plant response only when the vines are predisposed to stress (Gubler *et al*., 2000; Whiting *et al*., 2001). Recently, the three causal organisms have been recovered from inoculated 4-month-old pruning wounds (Gubler *et al*., 2001) and from spore traps in some vineyards in California (Eskalen and Gubler, 2001) while the presence of *P. inflatipes* in the soil has been confirmed by nested-PCR (Eskalen *et al*. 2001). The disease is also gaining importance worldwide, yet meager information is available on the mode of penetration of the causal organisms and the establishment of infection inside the host (Pascoe and Cottral, 2000). Such information is relevant to future studies on the effect of factors such as stress on host-pathogen interaction and on disease control. The present work was conducted to determine whether injury is a pre-requisite to penetration and to ascertain the path of invasion of *Phaeoacremonium* spp. in the roots and shoots of tissue-cultured grape plants.

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Materials and methods

Fungi and plant material

Inoculum of *P. inflatipes* and *P. aleophilum* was prepared from two-week old colonies growing in potato-dextrose agar (PDA) amended with 0.1 μg ml^{-1} tetracyline (PDA-tet). In all experiments, inoculum was used at concentrations of approximately 1×10^6 spores ml^{-1} of water.

Plants were obtained by aseptically growing single-bud cuttings of grapevine cv. Cabernet Sauvignon in culture tubes containing liquid or solid Murashige and Skoog culture medium or in Magenta vessels containing solid medium. For liquid culture, filter paper bridges were used as support. The plants were maintained in a growth chamber (18°C, 12-h light) before inoculation and on the laboratory bench (23±2°C, natural daylight) after inoculation.

Inoculation techniques

Inoculation of uninjured roots and shoots for penetration study

Two methods were used for inoculating roots. Roots growing in culture tubes with liquid medium were inoculated by replacing the medium with inoculum for 30 min. Enough spore suspension was poured into the tube to cover the entire root. Roots growing in culture tubes with solid medium were inoculated by injecting 0.5 ml of the spore suspension into the medium around the intact root. For shoot inoculation, plants growing in Magenta vessels were used. In this container, plants could be conveniently inoculated without uprooting them, hence avoiding the risk of mechanical injury. Plants were inoculated by spraying the inoculum on the stem, petiole and lower leaf surfaces with an atomizer. Samples for isolation and scanning electron microscopy (SEM) examinations were taken at 24-h-intervals for 7 days.

Inoculation of injured roots and shoots to determine the path of fungal invasion

Plants growing in Magenta vessels were used. For root inoculation, plants were carefully removed and placed on top of sterile paper towels. About 1.5 cm of the root tip was cut and discarded and the cut root base was dipped in the spore suspension for 30 min. Afterwards, the inoculated plants were carefully planted back in the medium. Shoots were inoculated by cutting the shoot tip and depositing a drop of inoculum (approximately 10 μl) on the wound. Samples for isolation and light microscopy observations were taken two weeks later.

Isolation

Samples were cut in small pieces and surface-sterilized by dipping them in 0.5% NaOCl for 3 min. The samples were rinsed twice with sterile distilled water, placed on PDA-tet and incubated at room temperature for 10 days.

Scanning electron microscopy

Sample pieces were fixed in 5% gluteraldehyde in $NaH_2PO_4 \cdot H_2O$ phosphate buffer (pH 7.0) and dehydrated with increasing concentrations of ethanol (10% interval from 10 to 100%, for 30 min each). Samples were then critical-point dried, mounted in aluminum stubs, sputter-coated with gold and examined in an electron microscope.

Light microscopy

Sample pieces were fixed in formalin-alcohol-acetic acid solution, then dehydrated with increasing concentrations of ethanol and infiltrated with paraffin using an automatic tissue processor. The schedule was as follows: EtOH solutions at 10% increments from 50 to 80% for one h each, 95% EtOH containing 1% Eosin for 1 h, two changes of 100% EtOH for 2 h each, 2 changes of Histoclear (CitriSolv) for 2 h each, histoclear with paraffin chips for 4 h, and 2 changes of paraffin for 4 h each. Samples were embedded in Paraplast Plus and cut to 10 μm sections using a rotary microtome. The sections were stained with safranin and fast green (Yilum *et al.*, 1993) and examined under a light microscope.

Results

Penetration of uninjured roots and shoots

The spores germinated on the root surface within 24 h and formed conidiophores and spores within 48 h (Fig. 1A), as viewed under SEM. Many spores formed germ tubes and spread over the root surface without showing signs of penetration. Penetration, when it occurred, was by the formation of numerous appressoria (Fig. 1B). On leaves, the germ tubes grew randomly, sometimes passing directly over open stomata. Penetration of stomatal pores was never observed (Fig. 2A) although ap-

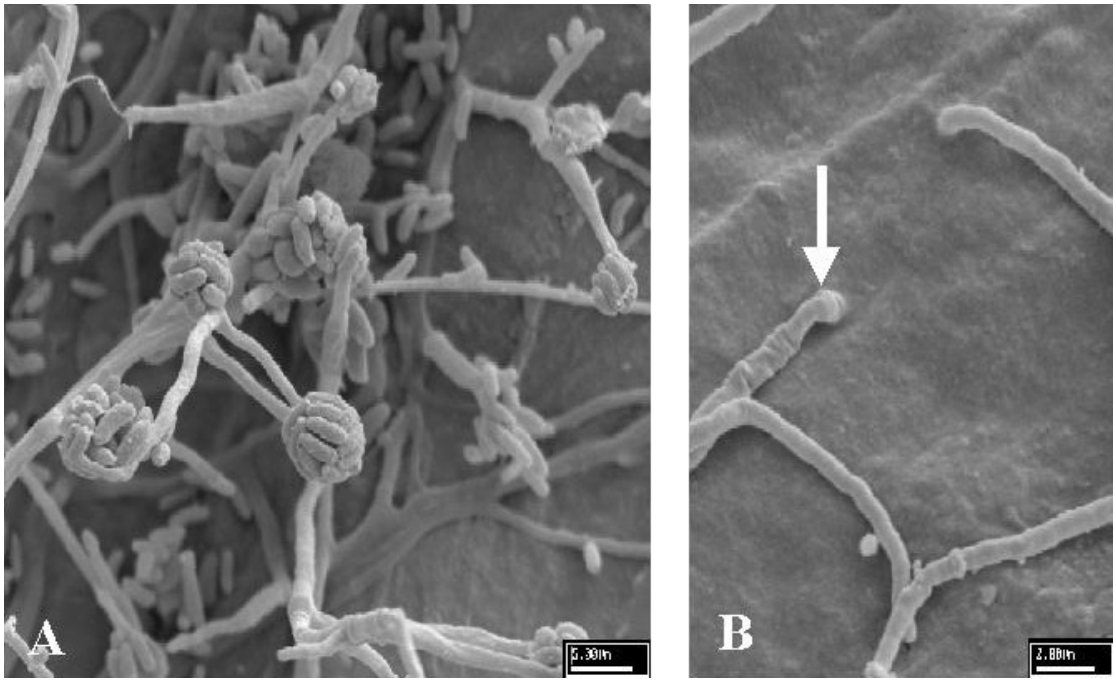

Fig. 1. SEM photomicrographs of *Phaeoacremonium inflatipes* on the root surface 48 h after inoculation showing (A) conidiophores and spores and (B) apparent hyphal penetration via appressoria (arrow).

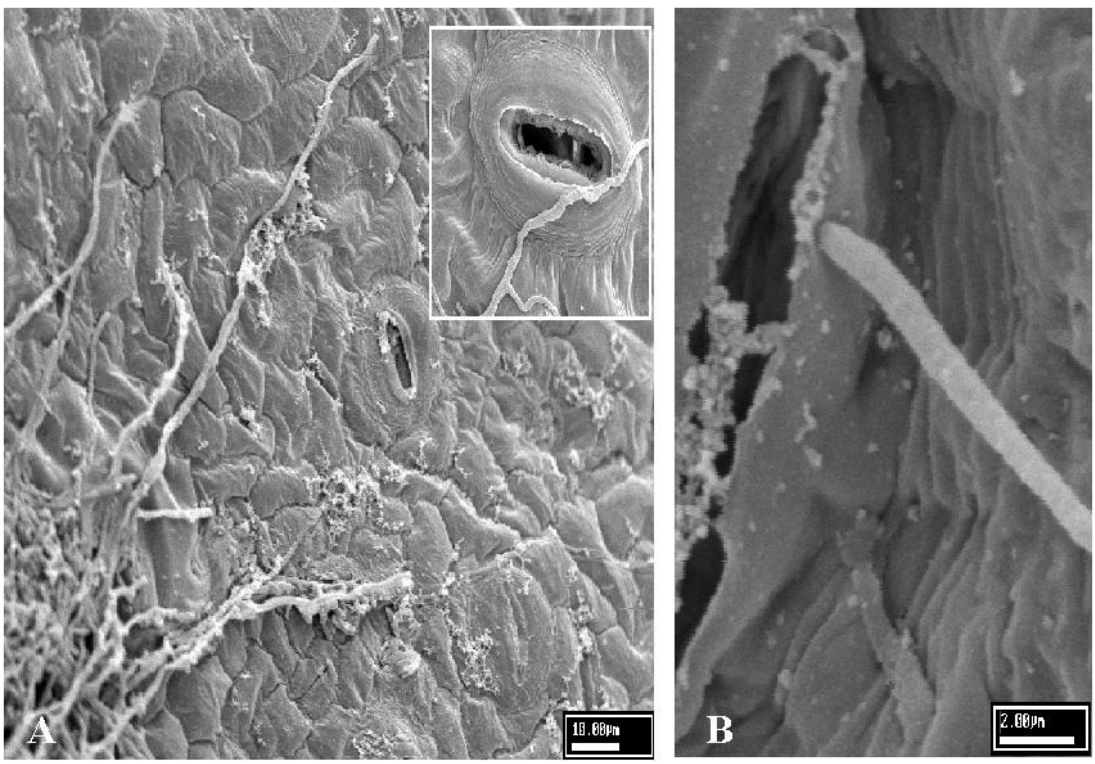

Fig. 2. SEM photomicrographs of the leaf surface 48 h after inoculation with *Phaeoacremonium inflatipes* showing (A) the fungus bypassing the stomatal pore and (B) apparent penetration of a guard cell of the stomata (B).

parent penetration of a guard cell was seen in one occasion (Fig. 2B). Positive isolations were obtained on PDA-tet for both inoculated roots and shoots.

Course of pathogen invasion in injured shoots

Two weeks after inoculation of injured shoot tips, the only symptom of infection was dark brown to black necrosis that extended from the point of inoculation down the stem for 5 mm. Shoot tips of the control plants, inoculated with sterile distilled water, remained green.

Light microscopy observations of paraffin sections of inoculated shoots showed that the course of invasion of the host occurred in a similar way for both organisms. At the point of inoculation, extensive hyphal development in the cells and intercellular spaces of all tissues was observed, particularly in the cortex and pith. Some portions of the cortex and pith were completely collapsed as evidenced by their compressed appearance and heavy staining with safranin. In the vascular tissues, some cells were filled to varying degrees with tyloses, gums and/or masses of mycelia.

Upon examination of stem sections remote from the inoculation point, it was apparent that spread of the fungus was initially through the intercellular spaces of the epidermis, cortex, and pith (Fig. 3).

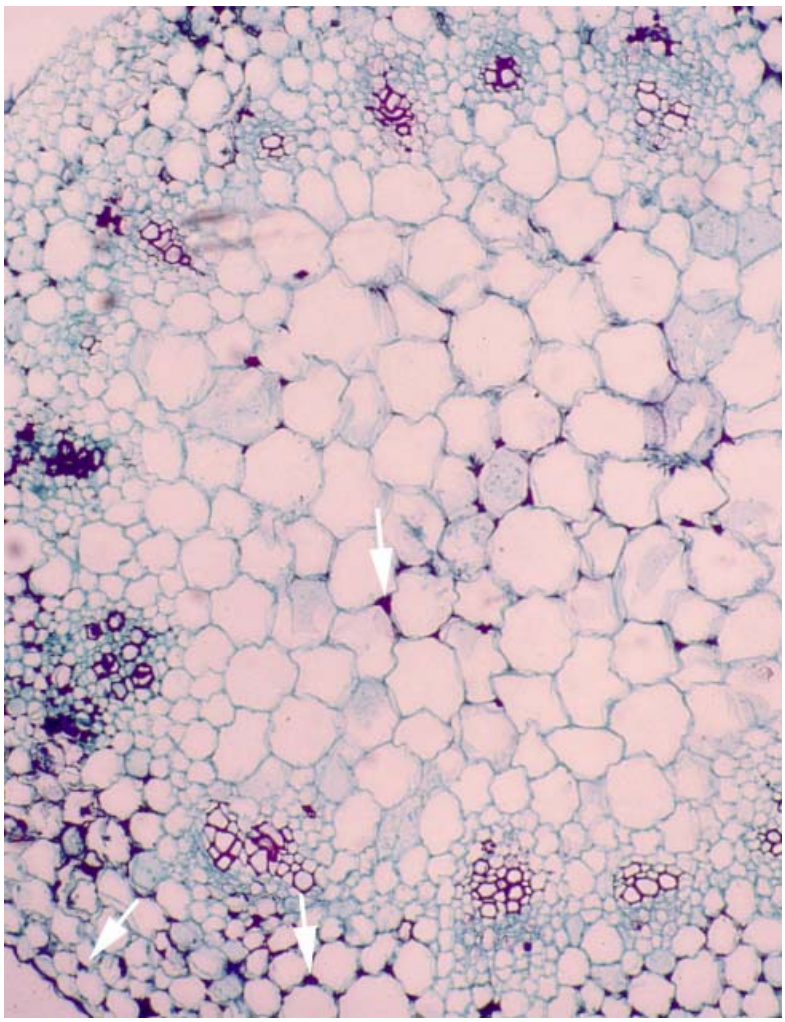

Fig. 3. Cross-section of an infected stem showing spread of the fungus through the intercellular spaces of the epidermis, cortex, and pith (arrows). ×=40.

Rapid spread seemed to occur in the pith where abundant inter- and intracellular hyphae were observed. Formation of conidiophores and spores was also observed in the intercellular space of the pith (Fig. 4)

The xylem and phloem were also invaded but the xylem more extensively. Numerous hyphal strands were observed spreading intracellularly in the xylem vessels (Fig. 5). Some vessels contained tyloses of different sizes and different amounts of gum deposits. Sometimes, gum deposits and hyphae were seen together in a single vessel element.

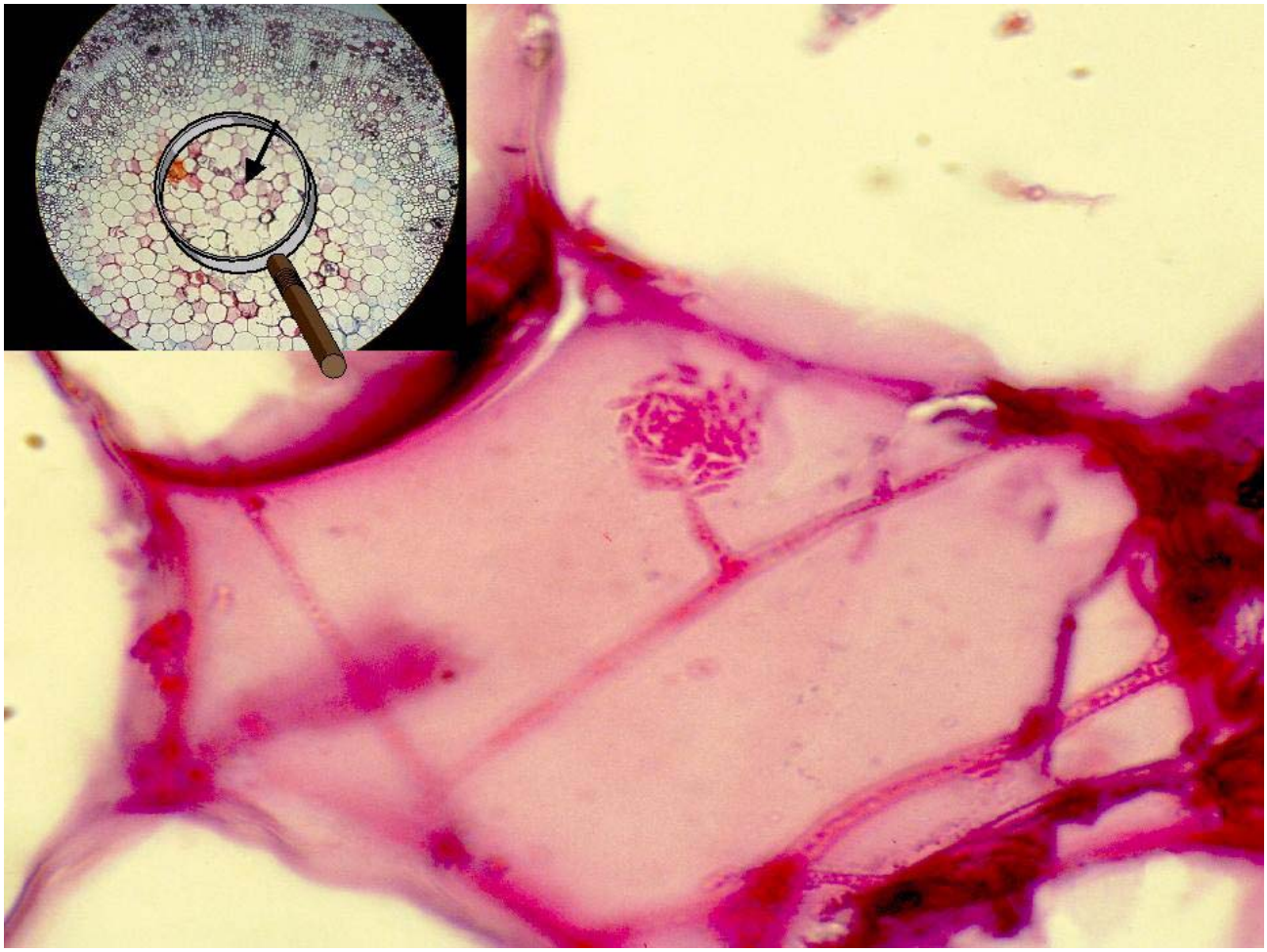

Fig. 4. Cross-section of an infected stem showing conidiophore and spores of *Phaeoacremonium inflatipes* in the intercellular space of the pith. ×= 1000.

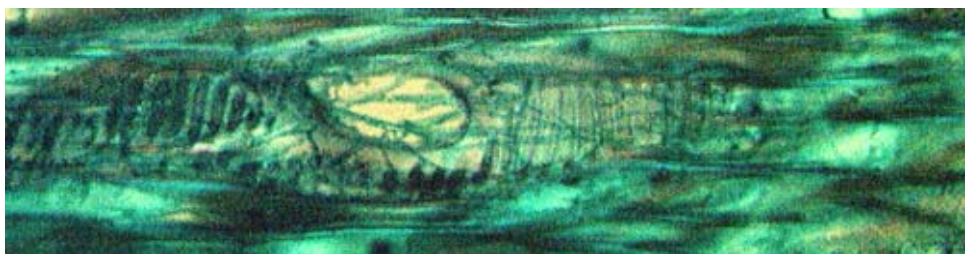

Fig. 5. Longitudinal section of xylem vessel with numerous hyphal strands. ×=1000.

In another section of the infected stem, heavy invasion of the secondary xylem was observed. In this section, numerous hyphal strands were present in the vascular rays and spores were detected in the metaxylem (Fig. 6).

Course of pathogen invasion in injured roots

Two weeks after inoculation, the roots showed brown discoloration that sometimes extended throughout their entire length. Microscopic examination of samples taken at the point of inoculation showed abundant hyphae in all the root tissues. Rapid spread to other areas was apparently accomplished by way of the vascular tissues and intercellular spaces. Further from the point of inoculation, the fungus was observed travelling in a longitudinal direction in the intercellular spaces of the outer cortical cells. Intracellular hyphae were seen occasionally, but most of the time the only indication of fungal presence was heavy staining of the intercellular spaces. Gum deposits or tyloses were observed in some vascular tissues, and occasionally both were present in a single vessel.

Reisolation of *Phaeoacremonium* spp. in inoculated plants

Typical symptoms of Petri disease were observed 2 months after inoculation of injured roots and shoots but isolations made on symptomless plants after 2 wk showed that the organisms were present in plant parts remote from the point of inoculation. Isolations were positive from shoots of root-inoculated- as well as from roots of shoot-inoculated-plants.

Discussion

The *Phaeoacremonium* spp. tested were capable of penetrating uninjured roots and shoots. Entrance into the shoot was apparently not through the stomata. Whether the organisms entered through other natural openings e.g. lenticels and hydathodes, or by direct penetration of the cuticle is not clear at the present time. The cuticle is generally regarded as an effective mechanical barrier to penetration by microorganisms, and thicker cuticles are frequently associated with in-

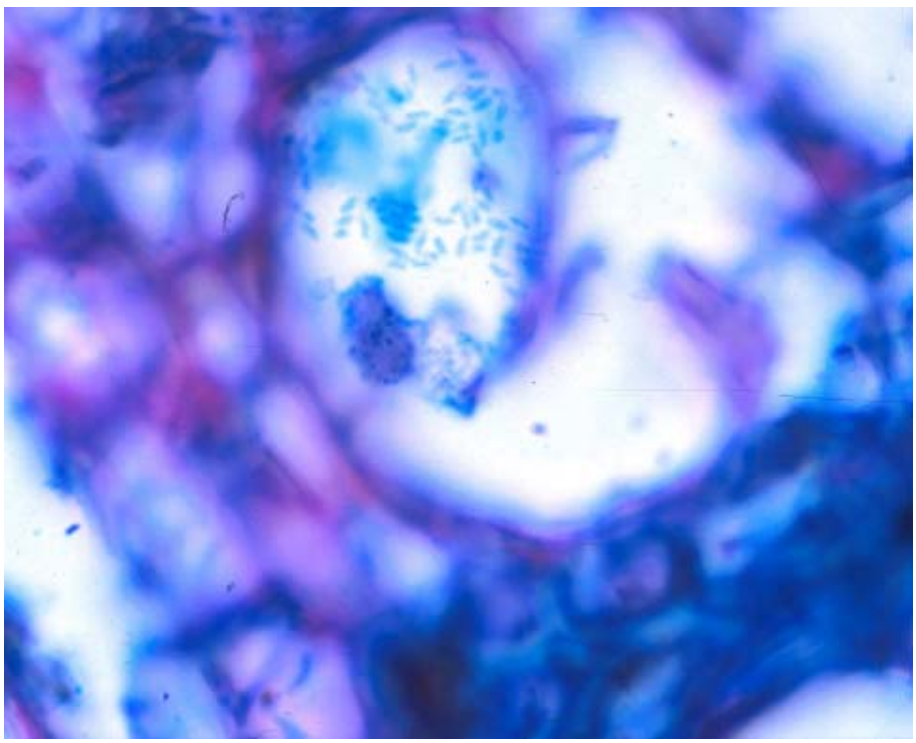

Fig. 6. Cross-section of stem showing fungal invasion in the secondary xylem. Note spores of *Phaeoacremonium aleophilum* in the metaxylem. ×=1000.

creased resistance to infection (Martin, 1964). Environmental conditions have been shown to affect cuticle thickness; plants grown under high humidity and under glasshouse conditions have thinner cuticles than those in the field and growing under low humidity (McNair, 1931; Skoss, 1955). Plants growing in the field are not expected to be as susceptible to penetration as the plants used in this study. Nevertheless, young shoots are regularly produced throughout the life of a grapevine and could be potential infection sites. Information on the penetration of uninjured aerial plant parts under field conditions is important particularly in light of the recent finding that spores of *Phaeoacremonium* spp. are disseminated aerially in California vineyards (Eskalen and Gubler, 2001).

This paper is to our knowledge the first report that documents the presence of spores in the xylem of grapevines infected with *Phaeoacremonium* spp. Although it has been suspected that rapid spread of the pathogen inside the host is accomplished by transportation of successive generations of spores through the vascular system, the presence of spores in the xylem has never been demonstrated visually though our laboratory has demonstrated that flowing sap contained spores of *Phaeoacremonium* spp. Plating of this sap showed the presence of conidia and colony growth on PDA-tet (Eskalen and Gubler, unpublished).

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