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ScienceDirect
Physiological and Molecular Plant Pathology 69 (2006) 209–223
PMPP
www.elsevier.com/locate/pmpp

# Effects of three esca-associated fungi on *Vitis vinifera* L.: I. Characterization of secondary metabolites in culture media and host responses to the pathogens in calli

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Accepted 30 April 2007

**Abstract**

*Phaeomoniella chlamydospora* (*Pch*) and *Togninia minima* (*Tmi*) produced scytalone, isosclerone and pullulans in liquid cultures, as well as in calli. Secondary metabolites and host defense compounds were shown to occur in calli of *Vitis vinifera* cv. Italia and cv. Matilde infected by the esca-associated fungi *Pch*, *Tmi* and *Fomitiporia mediterranea* (*Fme*). Calli of both cvs. were grown as dual cultures with *Pch*, *Tmi* and *Fme*. The fungi grew well in the presence of calli of both cvs., but callus growth was reduced. Accumulation and changes of total phenolics and recurring phenolics, and of two phytotoxic pentaketides and pullulans were analyzed by HPLC. On comparing results for cv. Italia and cv. Matilde, it can be seen that concentrations of phenolics are strongly related to the cv. The paper discusses the possible relationship between melanin biosynthesis in *Pch* and *Tmi*, which utilize pentaketide metabolites as intermediates and their pathogenicity related to phytotoxity of scytalone and isosclerone.


*Keywords:* *Vitis vinifera*; *Phaeomoniella chlamydospora*; *Togninia minima*; *Fomitiporia mediterranea*; Esca syndrome; Plant–pathogen interactions; Calli; Phenolics; Phytoalexins; Pentaketides; Pullulans

## 1. Introduction

Esca of grapevine (*Vitis vinifera* L.) is a complex disease whose symptoms may arise from the combined effect of several pathogenic and physiological factors [1–5]. The consistent isolation of the ascomycete *Togninia minima* (Tul. and C. Tul.) Berl. (anamorph: *Phaeoacremonium aleophilum* W. Gams, P.W. Crous, M.J. Wingfield and L. Mugnai) (*Tmi*), the anamorphic ascomycete *Phaeomoniella chlamydospora* P.W. Crous and W. Gams (*Pch*) and the basidiomycete *Fomitiporia mediterranea* M. Fisch. (*Fme*) from discolored or decayed wood of esca-diseased grapevines indicates a close relationship between those fungi and particular stages of wood, leaf and berry deterioration [2,4,6,7]. Pathogenicity of the three fungi was confirmed by inoculating them singly and in all possible combinations on the spurs, branches or trunks of standing esca-free cv. Italia and cv. Matilde vines. All three fungi were able to infect the vines through wounds, to colonize and degrade the woody tissue and, to cause disease symptoms very similar to those of esca [3,4,8]. The results of recent studies provided new information on the production of toxic metabolites by *Pch* and *Tmi* and host response compounds in naturally infected vines, indicating that the pathogens, their by-products and defense substances are translocated from the infected woody tissue of the trunk to the aerial part of the affected vines [9–11].

In many fungal diseases, phytotoxins are important virulence factors. Toxins can be host-specific or non-host specific. Phytotoxins are secondary metabolites, which have been isolated from artificial media rather than natural substrates, usually as a consequence of their accumulation in large and easily detected amounts. *Pch* and *Tmi* produced two pentaketides (scytalone and isosclerone) [12,13] and the α-glucan named pullulan [14]. These

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doi:10.1016/j.pmpp.2007.04.008

findings suggested that at least some of these metabolites may be involved in the pathogenesis and symptom expression of esca and related syndromes on grapevine. It would be interesting to know more about the roles played by those toxins, which are primary virulence factors, and the advantages (if any) they confer on the fungus which produces them.

It is now widely accepted that pullulan is a linear polysaccharide with malthotriosyl repeating units joined by α-(1 ⇒ 6)-linkages. Alternatively, the structural formula of pullulan may be presented as a regular sequence of panoses bonded by α-(1 ⇒ 4)-linkages [15]. The black yeast *Aureobasidium pullulans* (de Bary) G. Arnaud is widespread in all ecological niches including forest soils, fresh and sea water, plant and animal tissues, and is the main producer of pullulan [15]. *A. pullulans* is classified as a non-pathogenic microorganism; however, more recently the plant pathogenic fungi *Cryphonectria parasitica* (Murr.) Barr, *P. chlamydospora* and *T. minina* have also proved to be pullulan producers [14,16–19].

Resistance of plants to infection by plant pathogens is the result of multiple defense reactions comprising both constitutive and inducible barriers [20,21]. Active defense mechanisms mainly involve the accumulation of phytoalexins [22], rapid and localized cell death [23], synthesis of pathogenesis-related proteins [24], and reactive oxygen species (ROS) produced in the plant oxidative burst [25,26]. After infection, grapevines synthesize several compounds such as phytoalexins (e.g., the viniferins), phenolics and glycolic acid [9,27,28]. Synthesis and localization of these phenolics appear to be primarily associated with resistance against pathogens. Phytoalexins from the *Vitaceae* seem to constitute a rather restricted group of molecules belonging to the stilbene family, the skeleton of which is based on the *trans*-3,5,4′-trihydroxystilbene structure [24]. Flavonoids, phenolics with nuclei arranged in a $C_6$–$C_3$–$C_6$ configuration, are a diverse group of natural plant products, which play important roles in growth and development, and in defense against microorganisms and pests [29,30]. They are one of the largest groups of naturally occurring phenolics, which are likely to be encountered in any plant extract. Most flavonoids are glycosylated, and glycosylation makes them less reactive and more water soluble. Because of their *in vitro* antimicrobial activity, specific classes of flavonoid and isoflavonoid compounds have long been thought to play a role in plant–pathogen interactions as part of the host-plant defense arsenal [31].

In the present paper, much attention has been paid to the isolation of phytotoxins (pentaketides and α-glucans) produced by *Pch* and *Tmi* in batch cultures and in the infected calli of grapevine cv. Italia (more susceptible) and cv. Matilde (less susceptible). Moreover, the paper describes *in vitro* experiments on possible regulatory factors of the accumulation of phenolics in calli. This paper infers that the pentaketide pathway may be a wide-spread mechanism for the formation of fungal melanin.

## 2. Materials and methods

### 2.1. Fungal strains and culture conditions

Stock cultures of *P. chlamydospora* (*Pch*) strain PVFi56 (CBS 229.95), *T. minima* (*Tmi*) strain PVFi69 (CBS 631.94) and *F. mediterranea* (*Fme*) strain *Fop*1 isolated from grapevines in Italy were maintained on slants of malt-agar (MA) or potato-sucrose-agar at 4 °C. The three strains have been used since the pathogenicity trials carried out in 1999 [3,5,8].

All the strains were grown in stationary cultures in 1 litre Roux flasks containing 150 ml Czapek medium amended with 0.1% yeast and 0.1% malt extract (pH 5.7). Each flask was seeded with 5 ml of a suspension of three 10-day-old cultures in 50 ml sterile water. The flasks were incubated in the dark at 25 °C for 28 days. At harvest, the mycelial mat was removed by filtration on Miracloth (Calbiochem, La Jolla, CA, USA).

### 2.2. Dual cultures: fungus/callus

Explants of leaf petioles and young shoot internodes from cv. Italia and cv. Matilde grapevines were excised and cultivated both in Petri dishes and in Magenta vessels containing respectively 20 ml or 40 ml of a medium (LSG1) obtained by modifying Linsmaier and Skoog's medium [32] previously reported by Sparapano et al. [33,34]. Callus used for the experiments was produced after two transfers of 60 days each.

*Pch*, *Tmi* and *Fme* strains were grown in the dark at 25 °C for 2 weeks in Petri dishes containing MA. Plugs (3 × 3 mm) were aseptically removed from the margin of actively growing colonies and were transferred singly to Magenta vessels containing LSG1 medium with cv. Italia or cv. Matilde calli [34]. The plugs were placed in Magenta vessels parallel to the callus at a distance of 2 cm. All the Magenta vessels were sealed with Parafilm M (American National Can, Chicago, IL), and incubated in the dark at 25 °C. The experiment was carried out three times with three replicates per fungus. The growth of the colonies was measured every 4 days for 2 months. Calli were harvested when completely colonized by each fungus, their fresh wt was determined and then they were stored at −80 °C.

### 2.3. Extraction, purification and identification of phytotoxic pentaketides

#### 2.3.1. Batch cultures

The culture filtrates (3 litres per strain) were brought to pH 4 with 1 N HCl and extracted four times with ethyl acetate (1.5 litre each). The combined organic extracts were dried on anhydrous sodium sulfate and evaporated under reduced pressure to give a dark-brown oily residue. Chromatographic separation was performed on silica gel plates (Merk $F_{254}$, 0.50 mm, 20 × 20 cm) using a mixture of chloroform and ethyl acetate (3:1; v/v) as an eluent. Each