How iron could be involved in esca fungi development

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Summary. Iron reduction capability was detected by ferrozine assay in *Phaeomoniella chlamydospora*, *Phaeacremonium aleophilum* and *Fomitiporia punctata*. On the other hand, the Chrome Azurol S (CAS) assay demonstrated that low molecular weight chelator agents were produced by *P. chlamydospora*, *P. aleophilum*, *Libertella blepharis* and *F. punctata*. Foliar analysis carried out at harvest showed higher levels of the iron in symptomatic than in asymptomatic leaves. Conversely, levels of the other transition metals (copper, manganese and zinc), and of calcium and magnesium were basically similar in symptomatic and asymptomatic leaves. A possible relationship between iron, esca fungi and host-plant was hypothesized.

Key words: grapevine, siderophores, *Phaeomoniella chlamydospora*, *Phaeacremonium aleophilum*, *Fomitiporia punctata*.

Introduction

Wood deterioration in esca-diseased vines is very likely a result of both microbial processes (i.e. enzymatic activity, toxin spread) and vine/host reaction products (Mugnai *et al.*, 1999). Good *et al.* (1955) and Shigo and Sharon (1970) pointed out that the wood of hardwood trees deteriorated as part of a defence response to injury showed higher mineral levels than healthy wood. Shigo (1970, 1974) reported a correlation under laboratory conditions between concentrations of minerals (manganese, calcium, zinc and iron) and the mycelial growth of fungi associated with wood discoloration in maple, birch, beech and ash. The same Author suggested that these fungi were wound invaders because they had adapted to the high concentrations of minerals that are found in discolored and decayed tissues.

Mineral nutrients could well be involved in esca development in grapevine.

Several brown and white-rot fungi produce low molecular weight chelator agents (siderophores) with a metal chelating capability. Chelators reduce ferric iron for fungal metabolic functions. However, part of the reduced iron is released into the environment where it reacts with oxidants such as hydrogen peroxide to produce oxygen radical species, which in turn are active in the chemical oxidation of both cellulosic and phenolic compounds (Goodell *et al.*, 1997). On the other hand, the degradation of cellulose by enzymatic activity of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, the main micro-organisms involved in the early stages of esca, is a process that has not been completely elucidated (Mugnai *et al.*, 1997; Mazzullo *et al.*, 2000). Therefore siderophores pro-

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duced by esca fungi could be involved in the wood degradation process as well as enzymes, especially at those early stages.

For these reasons, laboratory tests are being carried out in order to explore a correlation between iron and the fungi associated with esca.

This paper reports on the behaviour of fungi in the presence of iron as well as on the detection of minerals in the leaves of esca-infected vines.

Materials and methods

Fungal strains

Fungi used in the tests were isolated from grapevines showing esca symptoms and maintained in 3.9% potato dextrose agar (PDA) at 20°C in the dark. *P. chlamydospora* strain 56 (CBS 229.95, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), *P. aleophilum* strain 4 (CSF, collection of DIPROVAL, University of Bologna, Italy) and *Fomitiporia punctata* strain CSF1.96 were used for the detection of ferrous iron. These fungal strains plus *Libertella blepharis* strain CSF1.97 and *Stereum hirsutum* strain LCP 93 2975 (P. Larignon, INRA, Bordeaux, France) were used to detect siderophores.

Detection of ferrous iron

Plugs (6 mm diameter) were cut with a cork borer from the margin of growing cultures and placed in the liquid medium of Higley (1973) modified by the inclusion of $Fe_2(SO_4)_3$ nH_2O (Farmitalia, Carlo Erba, Milano, Italy) at 50 μg ml^{-1} of iron instead of $FeSO_4$ $7H_2O$ at 0.015 μg ml^{-1} of iron. Carboxymethylcellulose sodium salt 0.5% (Fluka, c/o Sigma, St. Louis, MO, USA) was used as carbon source.

The detection of ferrous iron was according to Pierson and Clark (1984).

An aliquot of cultured liquid medium was collected at 24 days from inoculation and centrifuged for 5 min at 1500 *g*. The upper phase of the centrifuged medium (100 μl) was mixed with 100 μl of acid ferrozine solution (5.14 g ferrozine monosodium salt dissolved in 20 ml HCl 10% and diluted to 100 ml with bi-distilled water) in a safe lock tube, to which 750 μl of bi-distilled water was added. The tubes were boiled for 10 min. After cooling to room temperature, 100 μl ammonium acetate buffer (40 g ammonium acetate dissolved in 35 ml of 4% aqueous NH_3, and diluted to 50 ml with bi-distilled water) was added to the sample.

Serial solutions of $FeSO_4$ $7H_2O$ (Farmitalia) were made in the iron-free Higley medium to prepare a calibration curve.

Ferrous iron content was spectrophotometrically determined at 562 nm with a Jenway 6300 spectrophotometer (Jenway Ltd., Felsted, Dunmow, Essex, UK). Medium without iron was considered as a reference.

Analysis of each sample was performed in triplicate.

Detection of siderophores (Chrome Azurol S assay)

Chrome Azurol S (CAS) blue agar plates were prepared according to Fekete (1993). An amount of 60.5 mg CAS (Sigma) was dissolved in 50 ml water and mixed with 1 ml ferric iron solution (1 mM $FeCl_3$ $6H_2O$, 10 mM HCl). An amount of 72.9 mg hexadecyltrimethylammonium bromide (HDTMA) (Aldrich, Milwaukee, WI, USA) was dissolved in 40 ml water and slowly mixed with the CAS-Fe solution.

Separately, 30.24 g of piperazine were slowly added to MM9 salts dissolved in 750 ml water (1 X final salts concentration), and the pH was adjusted to 6.8 with 50% NaOH. Afterwards 1.5 g agar (Difco, Detroit, MI, USA) was added.

Both the CAS-Fe-HDTMA solution and the salt medium were autoclaved. The salt medium was cooled to 50°C before 30 ml of 10% casamino acids, vitamins and 300 μg ml^{-1} streptomycin sulphate (777 units base streptomycin mg^{-1}, Sigma) were added. The CAS-Fe-HDTMA solution and the salt medium were mixed at 50°C. The medium was poured into 90 mm Petri plates. Solid agar was overlaid with 10 ml XAD-4 adsorbent polystyrene (Sigma) in order to reduce the toxicity of HDTMA to the basidiomycetous fungi (Fekete, 1993). Inoculum plugs cut with a cork borer from the margin of growing cultures of *P. chlamydospora*, *P. aleophilum*, *L. blepharis*, *F. punctata* and *S. hirsutum* were placed in Petri dishes. Siderophores occurrence on CAS blue agar plates was shown by the formation of an orange halo around growing colonies.

Detection of iron, manganese, zinc, copper, calcium and magnesium from symptomatic and asymptomatic leaves

Both symptomatic and asymptomatic leaves

were collected at harvest from untreated 9-year-old vines (cv. Albana). Minerals were detected by the atomic absorpion spectrophotometric method (Cunniff, 1997).

Results

P. chlamydospora, *P. aleophilum* and *F. punctata* reduced the oxidized form of iron to a ferrous state. The ferrozine method showed that the amount of ferrous iron in the substrate was significantly greater for *P. aleophilum* (249 mOD), *F. punctata* (215 mOD) and *P. chlamydospora* (169 mOD) than for the control (153 mOD) (Fig. 1). The calibration curve indicated that 1 μg ml^{-1} of ferrous iron was approximatively equivalent to 20 mOD absorption.

Low molecular weight chelators were readily visible on CAS blue agar plates as indicated by the formation of an orange halo around the colonies of *P. chlamydospora*, *P. aleophilum*, *L. blepharis* and *F. punctata* (Table 1). Although the plates were overlaid with adsorbent polystyrene, HDTMA was toxic to *S. hirsutum* and inhibited its growth.

Before harvest, symptomatic leaves showed a higher amount of iron (228.8 mg kg^{-1}) than asymptomatic leaves (146.0 mg kg^{-1}). Levels of calcium, copper, magnesium, manganese and zinc did not differ substantially between symptomatic and asymptomatic leaves (Table 2).

Table 1. Siderophore production by fungi associated with esca as determined by Chrome Azurol S (CAS) assay.

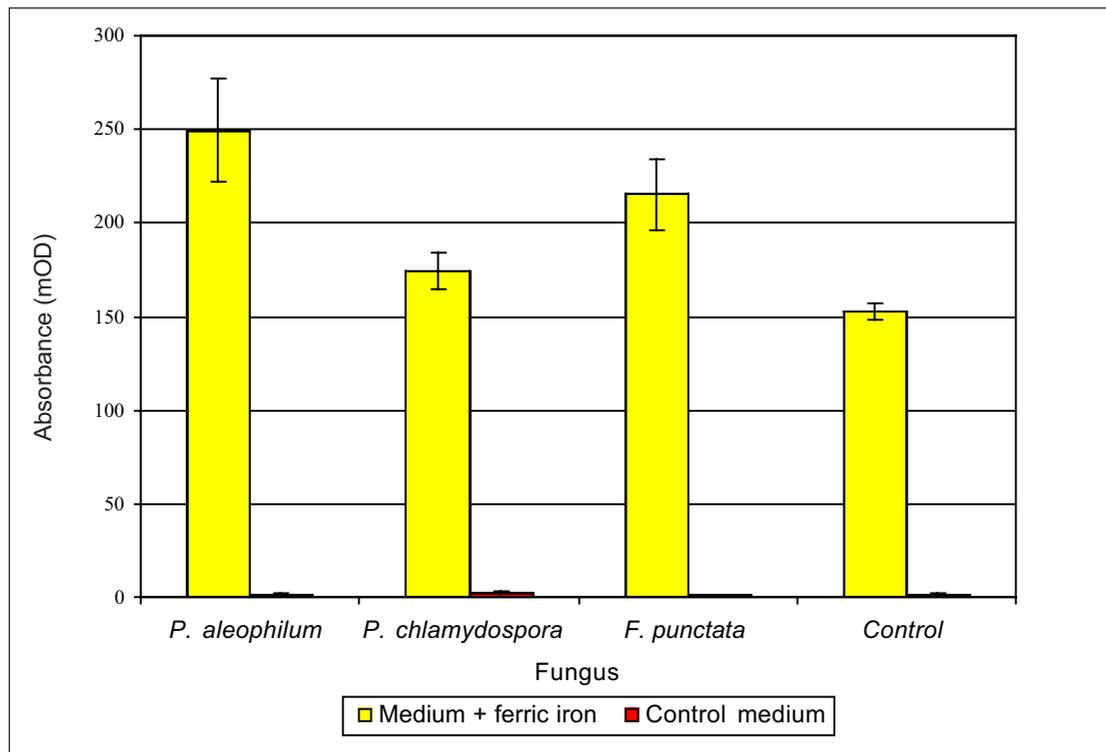| Fungal species	CAS assay
Phaeoacremonium aleophilum	positive[a]
Phaeomoniella chlamydospora	positive
Libertella blepharis	positive
Fomitiporia punctata	positive
Stereum hirsutum	no growth

[a] Positive, formation of orange halo around the colony.

Fig. 1. Ferrous iron concentration in slighly modified Higley liquid medium after growth of esca-associated fungi, *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum* and *Fomitiporia punctata*. Samples were collected at 24 days from fungal inoculation. Bars represent standard deviation.

Table 2. Mineral content (mg kg^{-1}) of symptomatic and asymptomatic leaves of grapevine infected with esca, as determined by atomic absorption spectrophotometry.

Mineral	Leaf	
	Symptomatic	Asymptomatic
Calcium	31,100 [a]	31,200
Copper	413	464
Iron	229	146
Magnesium	2,839	3,073
Manganese	120	113
Zinc	28	21

[a] Values are the means of 3 replicates.

Discussion

Our findings suggest that the three main fungi involved in esca, *P. chlamydospora*, *P. aleophilum* and *F. punctata*, reduce ferric iron to its reduced state, and that reduced iron in turn has a role in the complex relationship between these fungi and grapevines. Studies now under way on fungal degradation of cellulosic compounds in the presence of iron (unpublished data) seem to lend weight to this hypothesis.

Further studies are needed to investigate the correlation between fungus, mineral nutrients and grapevine defence response; nevertheless iron reduced by fungal siderophores and released into the environment could well be involved in the oxidation of both cellulosic and phenolic compounds (Goodell *et al.*, 1997).

Moreover, the ferrous iron could also become available for the plant (i.e. translocated). This hypothesis might explain why iron levels were higher in symptomatic leaves.

The role of iron in the degradation of cellulosic compounds and stilbenes by esca fungi is now being evaluated. The ultimate aim is to find applications of nutrients that will reduce the severity of esca.

Literature cited

Cunniff P., 1997. *Official Methods of Analysis of AOAC International. Supplement 1*. AOAC International Ed, Gaithersburg, MD, USA.

Fekete F.A., 1993. Assays for microbial siderophores. In: *Iron Chelation in Plants and Soil Microorganisms* (L.L. Barton, B.C. Hemming, ed.), Academic Press, Inc. San Diego, CA, USA, 399–417.

Good H.M., P.M. Murray and H.M. Dale, 1955. Studies on heartwood formation and staining in sugar maple, *Acer saccharum* Marsh. *Canadian Journal of Botany* 33, 31–41.

Goodell B., J. Jellison, J. Liu, G. Daniel, A. Paszczynski, F. Fekete, S. Krishnamurthy, L. Jun and G. Xu, 1997. Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *Journal of Biotechnology* 53, 133–162.

Highley T.L., 1973. Influence of carbon source on cellulase activity of white-rot and brown-rot fungi. *Wood and Fiber* 5, 50–58.

Mazzullo A., S. Di Marco, F. Osti and A. Cesari, 2000. Bioassays on the activity of resveratrol, pterostilbene and phosphorous acid towards fungi associated with esca of grapevine. *Phytopathologia Mediterranea* 39(3), 357–365.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83(5) 404–418.

Mugnai L., G. Surico and A. Sfalanga, 1997. Produzione di enzimi esocellulari da parte di funghi del legno di viti colpite da "mal dell'esca". *Micologia italiana* 1, 11–22.

Pierson E.E. and R.B. Clark, 1984. Ferrous iron determination in plant tissues. *Journal of Plant Nutrition* 7, 107–116.

Shigo A.L., 1970. Growth of *Polyporus glomeratus*, *Poria obliqua*, *Fomes igniarius* and *Pholiota squarrosa-adiposa* in media amended with manganese, calcium, zinc and iron. *Mycologia* 62, 604–607.

Shigo A.L., 1974. Effects of manganese, calcium, zinc, and iron on growth and pigmentation of *Trichocladium canadense*, *Phialophora melinii*, *Hypoxylon rubiginosum*, *Daldina concentrica*, and *Cytospora decipiens*. *Mycologia* 66, 339–341.

Shigo A.L. and E.M. Sharon, 1970. Mapping columns of discolored and decayed tissues in sugar maple, *Acer saccharum* Marsh. *Phytopathology* 60, 232–237.