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Antifungal effects of iron sulfate on grapevine fungal pathogens

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ABSTRACT

The present study aimed to determine the most efficient experimental conditions of iron sulfate use leading to optimal inhibition in the development of fungal pathogens. Assays have been focused on fungal species inducing severe grapevine diseases. $FeSO_4$ directly inhibited the *in vitro* mycelial growth of *Botrytis cinerea*, *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, *Diplodia seriata*, and *Neofusicoccum parvum* with variable efficiency in the range of 0.5–10 mM. The development was always completely inhibited at 20 mM. This inhibitory effect was greatly increased at acidic pH values. The anionic moiety of the molecule was of importance since bromide, chloride and sulfate were highly active, whereas acetate and oxalate showed a small effect. Electron microscope observations on *E. lata* and *B. cinerea* showed that a treatment with $FeSO_4$ induced dramatic changes in the hyphal organization leading to cell death. No toxicity was observed on grapevine leaves following repeated $FeSO_4$ sprays in the antifungal concentration range. Therefore, $FeSO_4$ may be proposed to effectively replace the long-term pollutant use of $CuSO_4$ as an antifungal agent, with the additional advantage of iron being an important plant micronutrient.

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1. Introduction

The aim of this research was to test the possibility that iron salts may be used as potential antifungal agents on the basis of known effects of heavy metals as antimicrobial agents. In particular, copper has been widely used for a long time in the well known "Bordeaux mixture" to control fungal infection in plants (rose trees, many fruit trees) and more particularly in grapevines. *In vitro* assays showed that $CuSO_4$ reduced the fungal development of many fungi (Toppe and Thinggaard, 2000; Levinskaité, 2001; Borkow and Gabbay, 2005) by acting at several levels on the fungal cell metabolism. Thus, copper toxicity occurs through the displacement of other essential metals from their native binding sites or through ligand interactions (Borkow and Gabbay, 2005). Exposition to elevated copper concentrations induces a disruption in membrane integrity, causing in particular a selective change in the plasma membrane permeability leading to loss of cell viability (Ohsumi et al., 1988; Borkow and Gabbay, 2005). Furthermore, the activity of enzymes and transport systems for essential nutrients and ions is inhibited through interaction with reactive sites in relation with the metal affinity to sulphydryl and other thiolate groups (for review, see Gadd, 1993). In addition, copper catalyzes the generation of deleterious free radicals (Stohs and Bagchi, 1995; Borkow and Gabbay, 2005).

But, the extensive use of the Bordeaux mixture for more than a century by grapegrowers has resulted in soil pollution in many grapevine areas (Fernandes and Henriques, 1991; Brun and Geoffrion, 2003). Therefore, we examined whether iron, a metal of the same chemical series, could be a surrogate of copper. The first essential step to address this issue is to determine the possible fungicidal action of iron. The importance of iron has been reported in the relationship between various micro-organisms and their hosts (Weinberg, 1966; Expert et al., 1996; Lemanceau et al., 2009). In particular, a pathogen attack triggered iron mobilization that enhances the production of reactive oxygen species in plant cells (Liu et al., 2007). Additionally, the experiments have been mainly carried out with sulfate salt on account of the fungicidal impact of sulfur (Cooper and Williams, 2004). Indeed, it has been reported that sulfur applied in the form of sulfate has a significant effect on the health status of plants (Klikocka et al., 2005) and that sulfur metabolites are involved in disease resistance, supporting the concept of sulfur-induced resistance (Bloem et al., 2005; and references therein).

Grapevines are infected by many fungal pathogens which cause severe diseases, therefore resulting in serious economic losses. In the course of this research, we focused our investigations on the possible antifungal effect of iron sulfate towards *Botrytis cinerea* Persoon:Fries, the agent of gray mold disease, and towards *Eutypa lata* pers. Fr. Tul. & C. Tul., the agent of dying arm disease, chosen

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as a model to study the wood-damaging diseases for which there is no available curative treatment.

B. cinerea is a ubiquitous pathogen fungus which attacks the leaves as shown by brown areas on the blade, the inflorescences and the berries which become brown and wilted. *E. lata* (initially named *E. armeniacea*) invades the vascular system of the trunks and shoots of many trees through pruning wounds, producing a characteristic dark wedge-shaped necrosis of the woody tissues leading to a wood decay classified as soft-rot (Rolshausen et al., 2008). In grapevines, after an incubation period of three or more years, the disease expresses dwarfed and withered shoots, marginal necrosis of the leaves, dryness of inflorescences and, finally, shoot death (Moller and Kasimatis, 1978). Our observations have been extended to other pathogens inducing similar events in other wood-damaging diseases. Thus, in esca in which *Phaeomoniella chlamydospora* P.W. Crous & W. Gams and *Phaeoacremonium aleophillum* W. Gams, P.W. Crous, M.J. Wingfield & L. Mugnai occur together in the early stages, leaf necrosis is observed and particular wood damage is seen as black streaks (Mugnai et al., 1999). Concerning Black Dead Arm (BDA), the leaf and wood symptoms of the disease have been reproduced following infection with *Diplodia seriata* De Not. and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips (Cristinzio, 1978; Auger et al., 2004).

At the present time, treatments of *B. cinerea* with a variety of fungicides have been long-term unsuccessful because of the rapid development of resistant strains in the field. More worrying is the fact that there is no efficient treatment for the control of the wood-damaging diseases, leading to important economic losses. Sodium arsenite had been used for many years to control esca but the withdrawal of this product (at the end of 2001 in France and sooner in other countries) abolished the only means of treatment. Therefore, any proposal able to replace this treatment would be of interest for the wine growers. However, in the wood-damaging diseases, one major problem is linked to the internal localization of the pathogens constituting an important impediment which should be carefully taken into account in all proposed treatments.

Thus, the aim of the present study was to assess the experimental conditions by which iron salts and, more particularly, $FeSO_4$ inhibit the vital processes of fungal development with maximal efficiency.

2. Methods

2.1. Fungal growth conditions

The fungi used were grown on a medium sterilized under 0.5 bars at 110 °C for 15 min and solidified with agar at 20 g l^{-1}. The growth medium was yeast nitrogen base minimal (YNBm, Difco, USA) at 1.7 g l^{-1} supplemented with glucose (10 g l^{-1}) and proline (5 g l^{-1}) according to Amborabé et al. (2005).

The strains of the different species were isolated from infected vines in vineyards from various areas. Concerning *E. lata*, the strain BI 1 was isolated near Poitiers (Octave et al., 2005), the strains NE 85 and NE 851-P in the Cognac area and the strain CM 96-7 in Burgundy. The various strains of *B. cinerea* (Bc 112 T, Bc 162 V, Bc 344 T, Bc 916 T), provided by Dr. Fermaud, were isolated in the Bordeaux area (Martinez et al., 2003). The strains of *P. chlamydospora* (PC-PC 3, PC-PC 21, PC-PC 32, PC-PC 37), of *P. aleophilum* (PA-PC 6, PA-PC 20, PA-PC 24), of *D. seriata* (BoF 99.6) and of *N. parvum* (BoF 00.21) were isolated in the Cognac area. The *P. aleophilum* strain PA-AQ 30 was isolated in the Bordeaux area. These strains were kindly provided by Dr. P. Larignon (IFVV Rodihlan, France) and Dr. J-P Péros (INRA Montpellier, France).

Mycelial growth was measured in the dark at 20 °C on cultures grown in Petri dishes containing the solid medium brought to pH 4.8 (except when otherwise indicated) with 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES)/KOH buffer. $FeSO_4 \cdot 7H_2O$ and other tested salts were added as a powder thoroughly mixed during cooling of the culture medium (at 50 °C) to obtain the desired final concentration. The inoculum consisted of a 3-mm diameter mycelial disk taken from a solid culture by means of a cork-borer. The diameter of the colony was measured daily in twelve identical wells for a period corresponding to complete mycelial invasion of the dishes in the controls. For each type of assay, experiments were carried out at least three times.

2.2. Electron microscopy

Samples were taken from the solid culture medium near the border of the colony and treated as previously reported (Valtaud et al., 2009). Briefly, samples were fixed for 45 min at room temperature in 1.5% paraformaldehyde/0.5% glutaraldehyde buffered at pH 7.3 with 0.05 M Sörensen buffer. After washings in the buffer containing 7.5% sucrose, they were postfixed in 1% osmium tetroxide for 5 min. Dehydration in increasing concentrations of ethanol (22, 50, 70, 95 and 100% for 10 min) was followed by embedding in LR White resin. Thin sections (70 nm thick) were collected on gold grids and stained with uranyl acetate and lead citrate for examination by transmission electron microscopy (TEM) (Jeol JEM 1010) at 80 kV.

2.3. Treatment on plant material

Two year old cuttings of *Vitis vinifera* L. cv. Cabernet Sauvignon were grown in a greenhouse under natural day-light conditions and fed daily with Snyder solution. The possible toxic effect of a treatment with iron sulfate was assayed by spraying blades (15 per assay) on standing vines with the tested salt solution supplemented with a wetting agent (Etaldyne 95, Rhone-Poulenc) at 0.5 ml l^{-1}. This effect was also assayed on mature leaves (15 per assay) excised under water from canes grown on these cuttings. The petioles were dipped in Eppendorf vials containing distilled water. These leaves were maintained in climate-controlled chambers at 25 ± 0.5 °C and 65 ± 5% relative humidity. Illumination was regulated so as to give 16 h of light (photophase 06.00-22.00 h) provided by fluorescent tubes (mixing Osram fluora and Osram day-light types) with a photon flux density (400–700 nm) of 36 μmol m^{-2} s^{-1} at the blade level. The possible occurrence of leaf necrosis was observed for 20 days after the beginning of the treament. In each protocol, the experiments were repeated three times.

2.4. Chemicals

Buffers and metal salts were purchased from Sigma–Aldrich Chimie (St Quentin Fallavier, France) and chemical products for electron microscopy from Euromedex (Souffelweyerssheim, France).

3. Results

3.1. Growth inhibition of E. lata by various sulfate salts

Results shown in Fig. 1 present the time course of mycelial development of *E. lata* in our experimental conditions. Thus, as seen on control sets, growth of the colony became measurable on the second day after inoculation and led to invasion of the culture well within 10 days. Sulfate salts of K and Mg slightly inhibited the fungal development, respectively 15 and 22% after a 8-d treatment, only affecting the growth rate. NH_4 sulfate showed a stronger effect inhibiting 60% of the colony development. However, this inhibition was only temporary since the growth rate was restored after

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