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Downy mildew: is resistance linked to inoculum concentration?

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Summary

Leaves of different *Vitis vinifera* cultivars, susceptible ('Chasselas' and '2185'), less susceptible ('2142') or resistant to downy mildew ('Solaris' and '2091'), were inoculated with four different concentrations of an aqueous sporangia suspension of *Plasmopara viticola* (5 × 105, 2 × 105, 6 × 104 and 2 × 104 sporangia·ml^{-1}). The infection rate of these samples was then examined by light microscopy and synthesis of stilbenes was analysed at infection sites. Infection rate increased parallel with inoculum concentration, but there was no correlation between the infection rate and resistance to *P. viticola*. Moreover, at the lowest inoculum concentration, the infection rate is similar for susceptible and resistant grapevine varieties. Quantification of stilbenes at 72 hpi showed that at the lowest inoculum concentration, the most susceptible grape variety synthesized the largest amount of stilbenes, whose level remained however below the ED50 values defined for each of them. Conversely, at the highest inoculum concentration, the most resistant varieties produced the highest amounts of the most toxic stilbenes against *P. viticola*. The critical role of the inoculum concentration used for artificial inoculation to evaluate grapevine resistance to downy mildew is discussed.

K e y w o r d s : inoculum concentration, *Plasmopara viticola*, resistance, stilbenes.

Introduction

Plasmopara viticola (Berk & Curt.) Berl. & de Toni, the causative agent of downy mildew, is one of the most widely distributed fungal diseases of grapevines worldwide. Given this fact and that most varieties of cultivated grapevines (*Vitis vinifera* L.) are susceptible to downy mildew, there is great interest in determining the mechanisms of resistance induced against this pathogen. For this purpose, different countries have initiated breeding programs to develop grapevine genotypes that are resistant to downy mildew. Therefore, a rapid method of estimating the level of resistance to downy mildew is needed to avoid long and tedious field observations.

Stilbenic phytoalexins are known to be key defence molecules and have been implicated in the resistance of grapevine cultivars to fungal pathogens such as *Plasmopara viticola* (Langcake 1981, Dercks and Creasy 1989, Pezet *et al*. 2003, Hammerschmidt 2004, Pezet *et al*. 2004 a, Alonso-Villaverde *et al*. 2011). Moreover, the quantification of stilbenes in grapevine seedlings after artificial inoculations with *P. viticola* sporangia has been shown to provide a good parameter to evaluate their level of resistance to this pathogen (Pool *et al*. 1981, Jeandet *et al*. 1992, Pezet *et al*. 2004 b, Gindro *et al*. 2006).

The aim of this work was to determine whether different concentrations of inoculum of *P. viticola* result in important differences in the resistance of five grapevine cultivars to the pathogen and to establish a concentration that yields reproducible defence responses by the grapevine cultivars.

Material and Methods

Cuttings of five *Vitis vinifera* cultivars that, according to Gindro *et al*. (2006), have different levels of resistance to *Plasmopara viticola* were used for this experiment, including the highly susceptible cultivars 'Chasselas', the susceptible '2185' ('Seyval blanc' × 'Gamaret'), the less resistant cultivar '2142' ('Gamaret' × 'Chambourcin') and the very resistant cultivars 'Solaris' ('Merzling' × ['Saperavi severneyi' × 'Muscat ottonel']) and '2091' ('Gamaret' × 'Bronner') as ranked before by Gindro *et al*. (2006). The numbered cultivars are issued from selections of the Swiss Federal Research Station Agroscope Changins-Wädenswil (ACW) breeding programmes. When rooted plants that were grown in a greenhouse (Pezet *et al*. 2003) reached the 10-leaf development stage, the 4th or 5th leaves from the apex were detached and placed in humid chambers.

The inoculum of *P. viticola* was obtained according to Gindro *et al*. (2003). Four different concentrations of an aqueous sporangia suspension were prepared: 5 × 10^5, 2 × 10^5, 6 × 10^4 and 2 × 10^4 sporangia·mL^{-1}. For each concentration of inoculum, three leaves per cultivar were inoculated with 20 µl droplets of the suspension.

At 24 hours post-infection (hpi), the sections of the leaf corresponding to the the infectious droplet surface at each inoculum concentration were collected for each cultivar. For each leaf, the infection rate [(number of infected stomata/total number of stomata) × 100] was scored for ten different visual fields (0.495 mm^2) using the KOH-aniline blue staining method (Díez-Navajas *et al*. 2007). Observations were made using an epifluorescence microscope (Leitz filter A (UV), excitation 340 nm, emission 380 nm, stop filter LP 430 nm).

At 72 hpi, three sections of leaf corresponding to the droplets surface at each inoculum concentration surface were cut for each cultivar and stilbenes were quantified by

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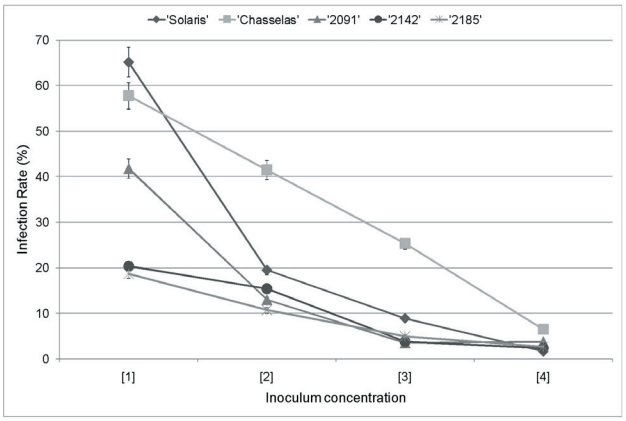

Fig. 1: Average infection rate (%) after artificial inoculations with *Plasmopara viticola* at the different inoculum concentrations applied: [1] = 5×10^5, [2] = 2×10^5, [3] = 6×10^4 and [4] = 2×10^4 (sporangia·ml^{-1}).

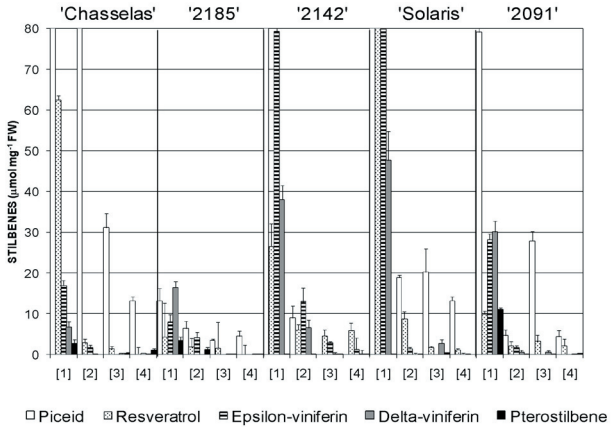

Fig. 2: Average quantification of stilbenes for the different cultivars studied, 72 hours post-infection with *P. viticola,* including standard error bars. The X-axis represents the different inoculum concentrations applied: [1] = 5×10^5, [2] = 2×10^5, [3] = 6×10^4 and [4] = 2×10^4 (sporangia·ml^{-1}).

UV-HPLC, as described by PEZET *et al*. (2004 b). The experiment was performed in triplicate, and the results are expressed as µmol·mg^{-1} fresh weight (FW).

Results and Discussion

At 24 hpi, when infections have successfully taken place, there is a positive correlation between the infection rate and the inoculum concentration for each cultivar tested (Fig. 1). Effectively, the infection rate increases as the inoculum concentration does. However, there is not a correlation between the infection rate and resistance to *P. viticola*. Indeed, 'Solaris', one of the resistant cultivars, exhibits the highest infection rate at the highest inoculum concentration (Fig. 1). Moreover, at the lowest inoculum concentration, the infection rate does not show significant differences between susceptible and resistant varieties. This means that resistance patterns are not defined by the infection rate and that there is not a direct correlation between resistance to the pathogen and the inoculum concentration. *P. viticola* is able to deregulate guard cell functioning (ALLEGRE *et al.* 2007), which could explain that high inoculum concentrations cause an increase of the infection rate. Therefore, this parameter is not a good indicator of the resistance of grapevines to this pathogen and cannot be used to determine resistance patterns. However, these results support that defence mechanisms are induced after infection takes place, such as the synthesis and deposition of callose, the production of reactive oxygen species (ROS), hypersensitive responses, peroxidase activity and the synthesis, accumulation and conversion of phenolic compounds, as described previously in the literature (DAI *et al*. 1995, GINDRO *et al*. 2003, KORTKAMP and ZYPRIAN 2003). The accumulation and conversion of stilbenic compounds from 48 to 72 hpi have been shown to be a good resistance indicator (POOL *et al*. 1981, DERCKS and CREASY 1989, PEZET *et al*. 2004 a, GINDRO *et al*. 2006, ALONSO-VILLAVERDE *et al*. 2011), when development of the pathogen is stopped in resistant varieties while intercostal mycelium and plenty functional haustoria are present on susceptible ones. Results from the quantification of stilbenes at 72 hpi (Fig. 2) show that 'Chasselas', at the lowest inoculum concentration, produces the largest quantity of the most toxic stilbenes, δ-viniferin (0.1 µmol·mg^{-1} FW) and pterostilbene (1.1 µmol·mg^{-1} FW) but insignificant according to the ED_{50} for these products (PEZET *et al*. 2004 a). Conversely, at the highest inoculum concentration, the most resistant cultivars, 'Solaris' and '2091', produce the highest amount of δ-viniferin (48 µmol·mg^{-1} FW) and pterostilbene (11 µmol·mg^{-1} FW), respectively. In addition the most susceptible cultivars, 'Chasselas' and '2185', synthesise respectively 7 and 3 times less δ-viniferin than 'Solaris' and 4 and 3 times less pterostilbene than '2091', respectively. The less resistant '2142' also produces ε and δ-viniferins, but in less quantities (79 and 28 µmol mg^{-1} FW respectively) and no pterostilbene. These results confirm previous results obtained by GINDRO *et al*. (2006), who analysed the different levels of resistance to downy mildew of these cultivars. These results also confirm that 'Solaris' produces mainly δ-viniferin and that '2091' mainly produces pterostilbene (ALONSO-VILLAVERDE *et al*. 2011). Therefore there is a better detection threshold and homogeneity between replicates when high inoculum concentrations are used rather than low inoculum concentrations.

The production and density of sporangia derived from the artificial inoculations are widely accepted as good criteria for estimating the resistance of grapevines to pathogens (DAI *et al*. 1995, LIU *et al*. 2003, SCHNEE *et al*. 2008). Particularly, BROWN *et al*. (1999) observed no differences in the sporulation of *P. viticola* following the artificial inoculation of different concentrations of a sporangia suspension, whereas other authors (DENZER *et al*. 1995, WIEDEMANN-MERDINOGLU *et al*. 2003) have observed that different inoculum concentrations have some influence on the sporulation of downy mildew. Sometimes, cultivars that appear to be resistant in the field are susceptible in potted grape cuttings in the greenhouse when sporulation is used as the criterion for resistance (GINDRO *et al*. 2006). This phenomenon is likely due to the more severe infection

conditions of artificial inoculations, which ensure an optimal pathogen development. In the field, even if one single zoospore is able to initiate the disease development, using a high infection pressure shows the true level of resistance of grapevine cultivars against *P. viticola*.

We conclude that, when biochemical criteria are used to evaluate the resistance traits of grapevine cultivars to downy mildew, the concentration of the inoculum plays an important role in the response of the plant. Moreover, the accuracy in quantifying stilbenes, which has been shown to be an essential criterion in evaluating the resistance of grapevines to *P. viticola* (Alonso-Villaverde *et al*. 2011), is directly related to the concentration of the inoculum, in which higher inoculums avoid diluting the stilbene signal detection in the quantification method.

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