Generation of antifungal stilbenes derivatives towards grapevine downy mildew using enzymatic secretome of *Botrytis cinerea*

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1 Introduction

Due to its polycyclic development, *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, the causal agent of grapevine downy mildew, requires numerous fungicide applications over the course of a season to control the disease to an acceptable level for high quality production. The use of repeated applications of single-site fungicides has led to the emergence of a higher frequency of resistant individuals in the pathogen population (Massi *et al.*, 2020). To limit the spread of the resistance phenomenon, one of the levers can be based on the discovery of new active substances with a different mode of action, to reinforce the panel of available active molecules, in a strategy of alternating application in the field.

Because of their heterotrophic nature, fungi colonize many ecological niches by synthesizing complex and protean enzymes in order to access carbonaceous substrates. In the case of fungal phytopathogens, the set of enzymes released into the extracellular space allowing infection in a host-pathogen interaction is defined as a "secretome". *Botrytis cinerea* Pers., an ubiquitous saprophyte attacking a very large number of plant species, has consequently had to develop an enzymatic arsenal to both establish the infectious process and detoxify possible host defense compounds. This implies a large panel of hydrolytic exoenzymes with cell-wall-degrading properties, considered as a factor of pathogenicity (Gonzalez-Fernandez *et al.*, 2015).

In response to a fungal infection, plants have developed strategies based in particular on the synthesis of antifungal substances that contribute to resistance. In grapevine, the stilbenic phytoalexin family, based on analogues of the precursor resveratrol, constitutes defense molecules against the main fungal diseases (Alonso-Villaverde *et al.*, 2011; Schnée *et al.*, 2013). However, stilbenes exhibit variable intrinsic antifungal activities, ranging from weakly active forms (glycosylated resveratrol) to highly effective analogues by oxidative dimerization or methylation.

In general, the exploration of natural products has greatly contributed to the identification of new antifungal substances in both the agronomic and medical fields. Due to their co-evolution, plants are producers of a wide variety of bioactive compounds whose structural diversity can confer a decisive advantage in the event of microbial infection (Dixon, 2001).

Several processes based on biological methods make it possible to obtain natural bioactive compounds. Among them biotransformation can be defined as the use of an intact whole organism or an isolated enzyme system to induce chemical modifications in organic compounds (Faber, 2004). If the biotransformation process is free of certain limitations encountered in classical organic chemistry (reactions possible under mild conditions, near neutral pH, ambient temperatures, and atmospheric pressure), it nevertheless comes up against some obstacles: i) the biotransformation using a given microorganism and by adding the compound to be transformed to the culture medium must have a low toxicity towards the microorganism used, ii) the purification process at the end of the reaction can be extremely complicated due to the mix of the generated metabolites and the microorganism metabolites, iii) the use of a purified enzyme to biotransform a natural product is limited by the small number of commercial enzymes available and their high cost.

In this context, an original method using the *Botrytis cinerea* Pers. protein secretome was developed to achieve the biotransformation of well-known grapevine stilbenic phytoalexins, naturally produced by resistant cultivars to limit the development of downy mildew (resveratrol, pterostilbene and a combination of both compounds) to generate unusual stilbene analogues with potent fungitoxicity. *Botrytis cinerea* was selected for the richness and diversity of its proteome, involved in pathogenicity and detoxification of defense compounds (Pezet *et al.*, 1991).

2 Materials and Methods

The secretome was obtained from a one-month-old liquid culture of *B. cinerea*, after filtration of the culture medium then precipitation and concentration of secreted proteins. The biotransformation of resveratrol, pterostilbene, and the mixture of the both compounds was performed using 10 mg of each pure compound, previously diluted in acetone. This solution was diluted in 200 mL of water and

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0.5 mL of the *Botrytis* secretome was added. This mixture was incubated at 22 °C in the dark, with agitation during 24h with samples taken at regular intervals to monitor the complete transformation of the substrates. The resulting crude reaction mixture was profiled by UHPLC-high resolution mass spectrometry (HR-ESI-MS), revealed the presence of compounds with unusual molecular formulae, suggesting the existence of new products. The biotransformation was scaled up to finally isolate and characterize 21 stilbene analogues by NMR and HR-ESI-MS analyses, respectively 7 specific compounds for the resveratrol and the pterostilbene biotransformation, and 12 for the mixture of the both compounds, some being redundant between the single and double subtracts reaction. The biotransformation of the two starting stilbenes allowed obtaining dimeric compounds and confirming the potential of this methodology to create chemical diversity of stilbene analogues (figure 1).

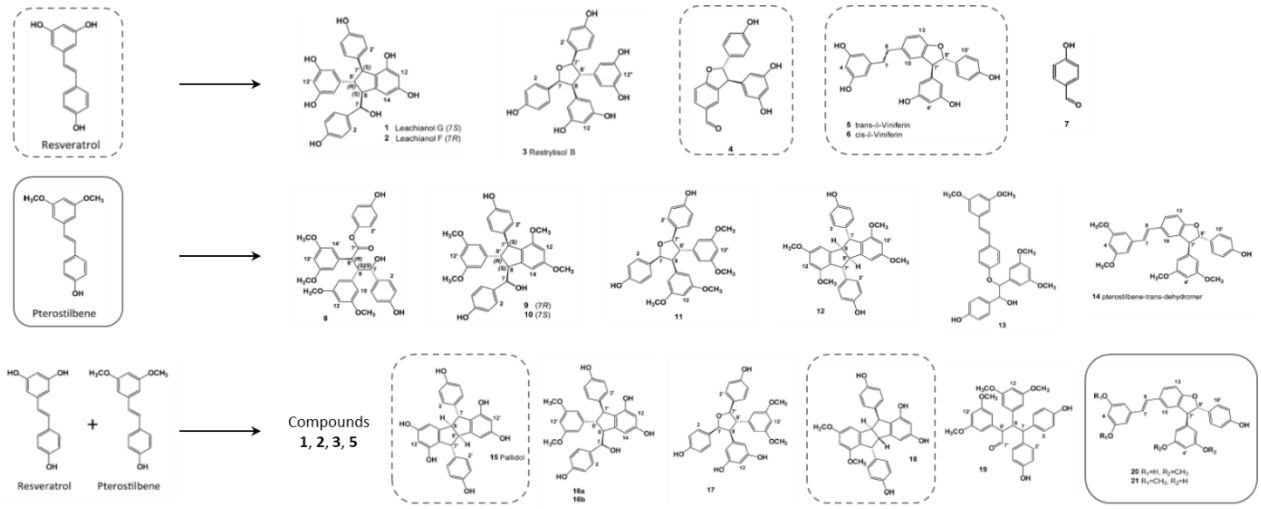

Figure 1: Compounds isolated from the biotransformation of resveratrol, pterostilbene and the combination resveratrol/pterostilbene. The antifungal activity of each compound is identified by the presence of a frame around the compound (absence: minimum inhibitory concentration (MIC) greater than 1 mM, dashed line: MIC between 1 mM and 10 µM, solid line: MIC less than 10 µM). Some minor compounds (8-13) in the reaction could not be tested due to their extremely low purified amount.

These generated compounds were evaluated for their antifungal properties against zoospores mobility of *P. viticola* and the cytological effects on the ultrastructure of *P. viticola* sporangia were observed by transmission electron microscopy (Schnee *et al.*, 2017).

3 Results

The biotransformed resveratrol compounds exhibit variable level of efficacy, some are not effective (1, 2, 3 and 7) whereas trans-δ-viniferin (5) is very toxic at low concentration, confirming data from previous studies (Pezet *et al.*, 2004). The biotransformation of pterostilbene, known to be a toxic molecule for downy mildew, generates products in too small amount to be tested, except the inactive pterostilbene-trans-dehydromer (14). The compounds 18 and 21 obtained from the enzymatic biotransformation of the combination of resveratrol and pterostilbene are very toxic against *P. viticola* zoospores mobility.

These results show interestingly that a moderately active starting substrate such as resveratrol can generate highly active compounds (4 and 6) through biotransformation, a process that seems to take place *in vivo* in resistant varieties under biotic or abiotic stress. In the same manner, a highly effective molecule, such as pterostilbene, can be converted into less toxic compounds (e.g., 14). The combination of these two initial molecules has permitted the generation of a wide range of stilbene analogues with variable antifungal level.

4 Conclusions

Biotransformation using the fungal secretome represents a promising strategy to generate new bioactive compounds with natural starting products in a green chemistry manner. The opportunity to create chemodiversity with enhanced bioactivity from conventional and available natural products allows higher throughput screening to target both agronomic and human pathogens.

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