Partial sequence of the *Erysiphe necator* cytochrome b gene

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Introduction

Powdery mildew, caused by the obligate fungus *Erysiphe necator* Schw., is a common and severe fungal disease of grapevine worldwide, due to the high adaptability of the pathogen to different climatic conditions. The disease control is exclusively depending on an intensive usage of fungicides in each season. The QoI-STAR (Quinol Outside Inhibitors - Strobilurin Type of Action and Resistance) fungicides is widely used to control grapevine powdery mildew. QoI-STAR fungicides it is synthetic derivatives of natural substances produced by basidiomycetes, such as *Strobilurus tenacellus* (Pers *ex* Fr) Singer. Because of their single-site mode of action, QoI-STARs are at high risk of resistance. Resistance has been so far detected in many phytopathogenic fungi.

The mechanisms of resistance to QoI-STAR fungicides involves one or several point mutations in the *cytb* gene, resulting in changes of the peptide sequence preventing fungicide binding (Zheng *et al*., 2000). At least 11 single or combined point mutations in the *cytb* gene were detected at amino acid position 127 to 147 and 275 to 296 (Baumler *et al*., 2003; Wood and Hollomon, 2003). The major mechanism of resistance is substitution of glycine with alanine at position 143 (G143A) of the cytochrome *b* protein (Gisi *et al*., 2000).

The development of suitable monitoring techniques and effective anti-resistance strategies are crucial to preserve the effectiveness of QoI-STAR fungicides. Duo to the obligate nature of *E. necator* resistance monitoring is difficult, time and labour-consuming. In the present work, baseline sensitivity to the QoI-STAR fungicides were determined and partial sequence of the *E. necator cytb* gene is obtained.

Materials and Methods

Fungicide testing. Leaves from *in vitro*-grown grapevine plantlets were used to assess the sensitivity of *E. necator* isolates to fungicides. Leaves production and maintenance of tested isolates were carried out as described by Miazzi *et al.* (1997). Commercial formulations of the QoI-STAR fungicides, azoxystrobin (Quadris®, Syngenta), were used. Fungicides were suspended in sterile water at the final concentrations of 0.1, 0.3, 1, 3, and 6 μg ml^{-1} of active ingredient.

In vitro produced grape leaves of cv. Baresana were used, taking care that they were uniform and of a similar size as much as possible to minimize any possible influence on the growth of *E. necator* colonies. Fungicides were applied just before usage by immersion of leaves into glass beakers containing the fungicide suspension at appropriate concentration for 1 min under gentle shaking; leaves immersed in water were the untreated check.

Single leaves were then placed in 55-mm-diam Petri dishes containing 10 ml of the substrate B0/2 Miazzi *et al.* (1997). Petri dishes were left closed in a laminar flow cabinet for 24 h before inoculation. Each leaf was inoculated on three points with 15-30 conidia. Leaves were then kept in a growth chamber at 21±1°C and exposed, 16 hours per day, to the light produced by a combination of 3 Osram L36W Cool White lamps and 3 Silvanya Grolux F36W lamps. At one week intervals, diameters of colony were measured with the aid of a stereomicroscope. When needed, resistance factor (RF) was calculated by using the formula RF = EC_{50} for the resistant isolate / EC_{50} of sensitive isolates; where EC_{50} is the Effective Concentration at 50%.

Molecular assay. The primers RSCBF1 and RSCBR2 ,designed by Ishii *et al.* (2001) on the ground of highly conserved regions of cytochrome *b* amino acid sequences in fungi and

proved specific for the *cytb* gene in *Sphaeroteca fusca*, were used. *E. necator* DNA extraction and amplification wer carried out as described by Miazzi *et al*. (2003). Amplification products were separated by gel electrophoresis as described by Miazzi *et al*.(2008). Amplified DNA from selected *E. necator* isolates was cloned and sequenced as described by Hajjeh *et al*., (2005).

Results

Twenty-five *E. necator* isolates sampled from 11 different Italian vineyards, with azoxystrobin treatment histories, were used to establish baseline sensitivity to azoxystrobin (QoI-STAR). EC_{50} values ranged from less than 0.1 to 0.3 μg ml^{-1} azoxystrobin, while colony growth was inhibited at 0.1 to 6 μg ml^{-1} of the fungicide. DNA amplification with the primers RSCBF1 and RSCBR2, specific for the *cytb* gene, responsible for resistance to QoI-STAR, yielded 2 bands having sizes of 286 bp (StI) and 224 bp (StII) (Fig. 1).

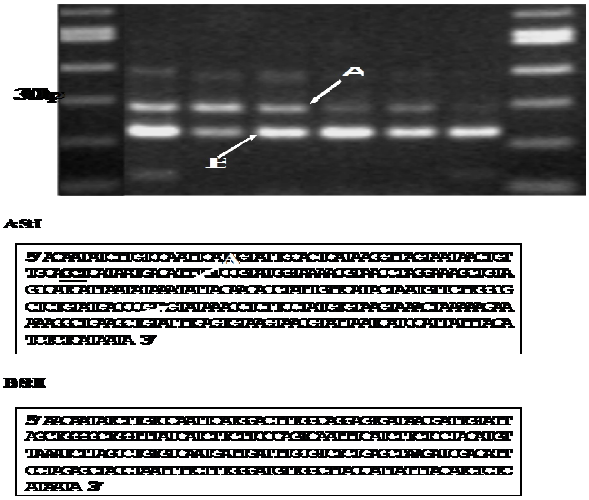

Figure 1 – Electrophoretic profiles obtained by amplification of *E. necator* DNA with the primers RSCBF1-RSCBR2, and sequences of St I (A) and St II (B)

Amplicons were cloned and sequenced as described by Hajjeh *et al*. (2005). DNA amplification, cloning and sequencing were repeated three times, to avoid any doubts on the correctness of the final sequences. FASTA analysis against sequences in Organelles Library of EBI GenBank, showed that StI, but not StII, had a high similarity with the sequences of the mitochondrial *cytb* of other fungi. The similarity within the best 100 scores was 68-87.7% identity and 69-87.7% for the ungapped alignment.

The StI nucleic acid sequence was translated into amino acid sequence using the ExPASy. The resulting amino acid sequence was aligned with those of other fungi; StI proved to contain amino acid sequence from 53 to 162 of the *cytb* gene (Fig. 2).

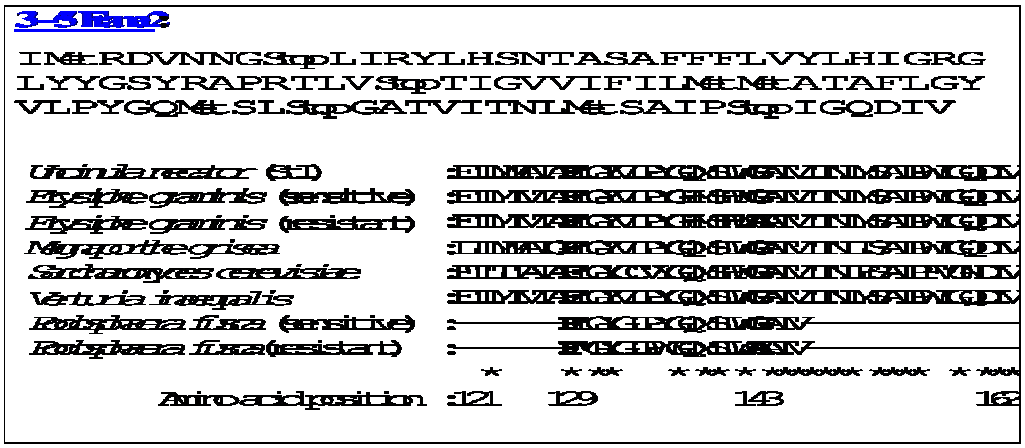

Figure 2 – StI translation (nucleic acid to amino acid; –2 shifted reading frame) and multiple alignments with the cytochrome *b* gene fragments containing the point mutations responsible of resistance to QoI-STARs in other fungi (in bold, position 129 and 143).

An *in vitro* technique was set up for assessing the response of *E. necator* isolates to fungicides. The technique allowed establishing baseline sensitivity of *E. necator* to azoxystrobin (QoI-STAR). The work herein discussed made a partial sequence of the cytochrome *b* gene of *E. necator* available; it will be helpful for further researches aiming at developing molecular methods useful for field monitoring of resistance.

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