NEW PERSPECTIVES FOR THE USE OF AMPELOMYCES-BASED BIOFUNGICIDES FOR EFFECTIVE CONTROL OF POWDERY MILDEW ON GRAPEVINE

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SUMMARY
Powdery mildew (PM) is one of the most widespread diseases of grapevine across Europe, and its control requires intensive fungicide sprays. During a 3-year study (2007-09) carried out in Northern Italy, the efficacy of late-season applications of the hyperparasitic fungus *Ampelomyces quisqualis* isolate M-10 (formulated product: AQ10® WG), a sustainable alternative to chemical fungicides, in reducing PM inoculum and thus disease pressure the following season was evaluated. AQ10 proved to be effective in stopping the production of new chasmothecia and reducing the number of mature ascocarps in potted grapevine plants, leading to reducing the PM severity on both leaves and bunches in the vineyard the following season. Late-season applications of AQ10 showed a mean efficacy of 40% (1 application) to 64% (2 applications) in reducing disease severity on bunches the following spring.

Key words: powdery mildew, grapevine, *Ampelomyces quisqualis*, AQ10, mycoparasitism.

RESUME
L'Oidium (*Uncinula necator*) est une des maladies de la vigne la plus répandue en Europe et son contrôle nécessite un nombre élevé de traitements. Une étude de trois ans (2007-2009) conduite en Italie du nord a évalué l'efficacité de contrôle d'un champignon hyperparasite, *Ampelomyces quisqualis* (isolat M-10 - Formulation AQ10® WG) appliqué en fin de saison. La formulation à base d'A. *quisqualis* peut en effet représenter une alternative durable aux fongicides chimiques en réduisant les spores d'Oidium et par conséquent la pression de la maladie la saison suivante. AQ10 a montré son efficacité en arrêtant la production de nouveaux chasmothecia et en réduisant le nombre d'ascocarpes mûres, conduisant à une diminution marquée de la pression de la maladie sur les feuilles et les grappes la saison suivante. L'efficacité moyenne obtenue avec la formulation AQ10 appliquée en fin de saison va de 40% (1 traitement) jusqu'à 64% (2 traitements) de réduction de sévérité de la maladie sur les grappes le printemps suivant.

INTRODUCTION

Powdery mildew is caused by *Erysiphe necator* (syn. *Uncinula necator*) (Schw.) Burr. and is one of the most widespread and economically important diseases of grapevine. A large number of fungicides are available for the chemical control of powdery mildew, including sulphur-based products, which have been applied against this target for more than one century. In European crop protection, the largest amount of fungicides has been used to control powdery mildews on various crops (Hewitt, 1998). Such an intensive application of fungicides is considered undesirable for both toxicological and ecotoxicological reasons, as well as for the risk of development of fungal strains resistant to the applied fungicides. The Fungicide Resistance Action Committee (FRAC, 2005) listed *E. necator* as a plant pathogen showing a medium risk of development of resistance to fungicides with different modes of action, including Quinone outside Inhibitors (QoI) (Wilcox et al. 2003), DeMethylation Inhibitors (DMI) (Miller & Gubler, 2003), and Aza-naphthalenes (Genet & Jaworska, 2009).

In the last decade, control of powdery mildew has become increasingly challenging because of the withdrawal of some active substances during the revision process under Council Directive EC 91/414 and the consumers’ request for a reduction of the number and amount of residues in the agricultural production. For these reasons, the EU is promoting the research for novel alternatives to chemical control in order to develop reduced-risk Integrated Pest Management (IPM) strategies and a more sustainable agriculture. An innovative approach to better manage plant diseases and rationalize chemical treatments is represented by the development of predictive models (Rossi et al., 1997). These models can be effectively combined also with applications of biofungicides to define alternative strategies for disease management. Recently, some mathematical models were successfully elaborated to improve plant protection strategies against powdery mildews on different crops such as sugar beet (Racca et al., 2000) and wheat (Rossi & Giosuè’, 2003). A mechanistic, weather driven model was also elaborated for the ascosporic infection of *E. necator*, which is widely considered a key element of powdery mildew epidemics (Caffi et al., 2010; Magarey, 2010).

Several studies demonstrated the importance of the overwintering inoculum of *E. necator*, represented by the ascocarps generated from the sexual reproduction, i.e. the chasmothecia (formerly cleistothecia), as an important source of inoculum for triggering epidemics the following season (Pearson & Gadoury, 1987; Gadoury & Pearson, 1990; Carisse et al., 2009). Rossi et al. (2010a) showed that chasmothecia formed at the end of the growing season can release ascospores in the same season, survive the winter, and discharge viable ascospores the following spring. Moreover, neither ascospore numbers nor their pattern of temporal release were influenced by the time when ascocarps had been formed (Rossi et al., 2010a).

An attractive and environmentally friendly alternative to control plant diseases is to apply natural enemies of the fungal plant pathogens, i.e. Biocontrol agents (BCAs), as formulated products (called biofungicides). BCAs of various powdery mildew fungi have been studied intensively in the past, and a review for greenhouse crops was prepared by Elad et al. (1996); these BCAs include bacteria, mychophagous arthropods, and fungi. Applications of bacteria and mychophagous arthropods did not provide satisfactory disease control, while more promising results were obtained with antagonistic fungi (Kiss et al., 2004). The hyperparasite fungus *Ampelomyces quisqualis* Ces. (Deuteromycetes, Dematiaceae) is one of the few fungi already available on the market as a biofungicide. *Ampelomyces quisqualis* can parasitize hyphae, conidiophores, and chasmothecia of more than 64 different species of powdery mildew fungi, including the genera *Brasilomyces*, *Erysiphe*, *Leveillula*, *Microsphaera*, *Phyllactinia*, *Podosphaera*, *Sphaerotheca*, and *Uncinula* (Falk et al. 1995; Kiss, 1998). The commercial biofungicide AQ10® WG (henceforth AQ10) contains at least $5.0 \times 10^9$ viable spores per gram of product of the isolate M-10 of *A. quisqualis*, which was
first identified on *Catha edulis* in Israel in 1986 (Szteinberg et al., 1989). The fungus is formulated in water dispersible granules (WG) by using a patented process (Hofstein et al., 1996). In 1999, Intrachem Bio Italia S.p.A. obtained a provisional registration of AQ10 in Italy, which was the first registration in Europe. Inclusion of the product into Annex 1 of Council Directive 91/414/EEC was achieved by the Intrachem Group in 2005. To date, the product is registered in Italy, Greece, Slovenia, South Africa, Spain, Switzerland, Germany, Cyprus, and Egypt, and registration is pending in France.

*Ampelomyces quisqualis* can be applied during different growth stages of the grapevine. Traditionally, the biofungicide was used in spring and summer against the asexually sporulating powdery mildew colonies, but reports on its efficacy are contradictory. Sometimes powdery mildew epidemics could not be controlled efficiently (SHISHKOFF & McGrath, 2002), while in other cases the application during the vegetative period provided a good efficacy in controlling powdery mildew, especially when applied in alternation with endotherapeutic fungicides (Zanzotto et al., 2005). Other studies also showed the potential of using the product during the late part of the season (pre- and post-harvest), with the aim of the fungus parasitizing the overwintering chasmothecia and replacing sulphur sprays to avoid any possible negative effects of sulphur on the fermentation process. Falk et al. (1995) obtained a reduction of the chasmothecia on the vine bark of up to 50 - 60% and a high percentage of parasitized chasmothecia by releasing *A. quisqualis* spores through colonized cotton-wick cultures suspended above the vines. Similar results were recorded in South Tyrol (Italy); pre- and post-harvest applications of AQ10 resulted in a significant reduction of the chasmothecia in comparison to an untreated control, even in the presence of a high level of natural parasitism by *A. quisqualis* (Haas et al., 2005). However, in spite of the fact that different authors reported the parasitism of chasmothecia by *A. quisqualis* (Gadoury & Pearson, 1988; Falk et al., 1995; Kiss, 1998), the mortality rate of the chasmothecia was never quantified (Jarvis et al., 2002).

Based on i) the key role played by the overwintering inoculum originating from the sexual reproduction of *E. necator* and ii) the ability of *A. quisqualis* to parasitize chasmothecia, a three-year study (2007-09) was carried out in Northern Italy in order to verify whether late-season applications of AQ10 on grapevine could reduce powdery mildew infection the following season. Studies were performed under both semi-field (greenhouse experiments) and open-field (vineyard experiments) conditions.

**Greenhouse experiments**

Green cuttings of grapevine cultivar Barbera, susceptible to *E. necator*, were grown in a greenhouse (at 18 to 26°C, under natural photoperiod) in 10x10 cm pots containing a mixture of sand, peat, and soil. Once the plants had reached the growth stage of at least 4-6 leaves unfolded (BBCH 14-16), the leaves were dry-inoculated with a mixture of *E. necator* conidia collected in untreated plots of different commercial vineyards in Emilia-Romagna (Northern Italy). Leaves were observed once per week in order to determine the initiation of the sexual reproduction of powdery mildew, consisting in the first development of young, pale yellow, chasmothecia. Once chasmothecia production had started uniformly on the grapevine leaves, 200 circles (Ø 2.5 cm) were drawn on eight different plants (mean of 5 circles per leaf) and the number of immature (i.e. yellow or brown) and mature (i.e. black) chasmothecia was counted. Four plants were then sprayed with the biofungicide AQ10, while additional four plants were sprayed with bi-distilled water alone, thus acting as untreated control. All plants were then incubated in saturated atmosphere for three weeks. Afterwards, the number of immature and mature ascocarps within the leaf circles was counted again. The increase of chasmothecia on treated and untreated leaves was then considered to quantify the efficacy of the biofungicide in reducing the development and maturation of the ascocarps. Four experiments were carried out at different incubation temperatures between 16 and 25°C.
In all the experiments, the biofungicide was able to completely stop the production of new chasmothecia. No new ascocarps were produced on the treated grapevine leaves within three weeks after treatment application. The number of mature chasmothecia was reduced by one- to two-third, depending on the experiment, and mean reduction amounted to approximately 40%. Therefore, parasitism by *A. quisqualis* of sexually reproducing powdery mildew colonies significantly reduced the formation and maturation of chasmothecia.

At the end of each experiment, some black chasmothecia were randomly collected from both treated and untreated grapevine leaves in order to evaluate the morphological maturity of the ascospores and their viability by using green fluorescence with the fluorescein diacetate stain. Neither maturation nor vitality of the ascospores produced into the chasmothecia treated with AQ10 were significantly affected by the treatment. This result is consistent with the mode of action of the hyperparasite fungus: *A. quisqualis* penetrates the host and forms picnidia within the ascocarps, thus preventing the formation of asci and ascospores (Jarvis *et al.*, 2002).

**Vineyard experiments**

Open-field experiments were carried out in six different vineyards in Emilia-Romagna (Northern Italy) in 2007/08 and 2008/09. In order to prevent ascocarp production, AQ10 was applied during the late part of the season using two different strategies: i) one application just after harvest, and ii) two applications, one before and one after harvest. The following season, none of the experimental plots (treated with the biofungicide and untreated) was sprayed against powdery mildew.

The following spring, all vineyards were carefully inspected at least once per week starting from bud burst (BBCH 08) to determine the time of appearance of the first disease symptoms, such as flag shoots or the typical discrete pale spots on the abaxial surfaces of the basal leaves caused by the ascosporic infections. Disease severity (% leaf/bunch area affected by powdery mildew) was assessed on a sample of 100 randomly selected leaves per plot three times between bud break and fruit set, and on 100 randomly selected bunches per plot at fruit set. Disease severity was estimated visually and expressed as a percentage of the total leaf (or bunch) area. Disease intensity (as a percentage) was then calculated as average disease severity of the sampled leaves (or bunches).

Flag shoots were never found during the vineyard surveys. Therefore, the primary inoculum of the disease was composed exclusively of the ascospores released by the overwintering chasmothecia. In the six study vineyards, disease intensity on leaves was low (maximum 6% of the leaf area affected) and treatments with AQ10 reduced the disease intensity by about 90% on average. In four vineyards, the disease developed on bunches, with 12 to 38% of intensity at fruit set. One post-harvest application of AQ10 showed a mean efficacy in reducing disease intensity on bunches by approximately 40%, and a maximum efficacy of more than 50%. When the biofungicide was applied twice (once pre-harvest and once post-harvest), instead, mean efficacy in suppressing disease severity on bunches amounted to 64%, and maximum efficacy exceeded 70%.

These results demonstrate the capacity of the hyperparasite to suppress the production of ascospores in the spring following late-season applications of AQ10 and, consequently, disease severity on both leaves and bunches. Highest reduction in powdery mildew severity was achieved when the *A. quisqualis*-based product was applied twice, with the first application being carried out before harvest. At that time, most of the chasmothecia are present in an immature stage (Rossi *et al.*, 2010b), and thus more susceptible to the penetration of the hyperparasite. It is known that *A. quisqualis* is more likely to parasitize the fruiting bodies during their early developmental stages, before the ascocarp wall darkens.
(Falk et al., 1995). Therefore, appropriate timing of the application of this BCA is of sound importance: it should be applied when the majority of the ascocarp population is still in the yellow maturation stage. A model predicting the maturation of the ascocarp population of E. necator in the vineyard could help growers in the accurate timing of the AQ10 applications (Rossi et al., 2009).

Further experiments are in progress to include late-season applications of the Ampelomyces-based biofungicide in a new IPM strategy, in which powdery mildew control is based on the inoculum level present in the vineyard.

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