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Combining biocontrol agents with different mechanisms of action in a strategy to control *Botrytis cinerea* on grapevine

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The use of several microbial biocontrol agents to combat *Botrytis cinerea*, the causal agent of grey mould, has been studied. However, only a few microorganisms have been developed as biofungicides, which are currently used in some countries, mostly in organic farming. The main reason for the limited market uptake of microbial biofungicides is their debated variable efficacy. To cope with poor survival in the canopy, due to unfavourable environmental conditions or their intrinsic lower level of disease control compared to synthetic chemical fungicides, use of a mixture of two or more microorganisms with different environmental requirements and mechanisms of action has been proposed with contrasting results. However, their use in strategies involving calculated timing of the microbial biocontrol agents, taking into consideration their mechanism of action in relation to the epidemiology and pathogenesis of the disease, has never been attempted in relation to combating grey mould on grapes. The results of four years of trials in three locations in Northern and Central Italy show that *Trichoderma atroviride*, *Aureobasidium pullulans* and *Bacillus subtilis*, applied at bunch-closure, veraison and pre-harvest, respectively, controlled *B. cinerea* on bunches very satisfactorily, and the results did not differ from those obtained with a strategy combining the three biofungicides, applied at the aforementioned stages. Colonisation of berries by each of the different microbial biocontrol agents at harvest time did not differ for individual treatments or when applied in the combined strategy, suggesting that the microorganisms did not negatively interfere with each other and that they may possibly occupy different ecological niches. The high level of efficacy of the tested biocontrol agents against grey mould can be explained with the relatively low-medium level of the disease, their integration with agronomic practices or the optimal timing of the treatment.

1. Introduction

Botrytis cinerea (= *Botryotinia fuckeliana*; Johnston et al., 2014) is an extremely polyphagous and ubiquitous pathogen and the causal agent of grey mould, one of the major diseases of the grapevine. On grapevine, it may cause significant losses in terms of quantity and quality, especially on sensitive varieties and when disease-conducive meteorological conditions prevail (Elad et al., 2007). Control of *B. cinerea* on various crops is commonly achieved with a combination of pesticide treatments and agronomic practices. On the grapevine, such practices can directly or indirectly influence the disease, by modifying both berry defence mechanisms and the microclimate of the vine. For example, avoiding excessive nitrogen fertilisation, removal of leaves around the bunches and thinning of the berries can significantly reduce the disease (Mundy, 2008; R'Houma et al., 1998). Removal of leaves in the fruiting zone increases their exposure to the sun, resulting in more epicuticular wax and a more resistant cuticle, and thanks to higher air-flow in the canopy, in a reduction of relative humidity and faster drying of the bunch following rain (Gubler et al., 1987). In addition, removal of leaves in the bunch zone at the 'pea-size berries' stage can reduce

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infestation by the second generation of *Lobesia botrana* (European grapevine moth) and consequently limit grey mould, which develops as a result of feeding damage caused by this insect (Pavan et al., 2016). Varieties with tight bunches are considered to be more susceptible to *B. cinerea*, not only because of compression among the berries, which can create wounds easily colonised by the pathogen, but also because of the intrinsically higher susceptibility of the epicuticular wax at the point of berry contact (Marois et al., 1986). The advantages of increasing bunch openness, achieved with the use of gibberellic acid, is still controversial (Ferree et al., 2003; Mundy et al., 2014) and bunch tightness should probably be considered in the wider context, being only one of the factors concurring in susceptibility to the disease in some varieties (Vail et al., 1998).

Botrytis cinerea infections may start at bloom, when the most likely site of infection is the receptacle area or the cap scar (Keller et al., 2003) and remain latent until after veraison. After veraison, the sugar concentration increases and antifungal plant compounds decrease, with a parallel increase in berry susceptibility to *B. cinerea* (Jacometti et al., 2010). *Botrytis cinerea* can easily colonise senescing floral tissues that remain trapped inside compacted bunches and result in a source of inoculum following veraison. In addition, *B. cinerea* germination is promoted by the presence of sugars, which may be exuded by ripening tissues or leached from micro and macro-wounds on the skin of ripening berries. Recent studies demonstrated that in some regions grape inflorescences are more susceptible at flowering (beginning, full, and end of flowering) than at earlier growth stages or at fruit swelling or berries groat-sized stages (Ciliberti et al., 2015).

Although very helpful, agronomic practices alone cannot prevent the disease in many grape-growing areas, so chemical treatments should normally be applied (Jacometti et al., 2010). Because of *B. cinerea* epidemiological traits (the inoculum is always present in the vineyard and the range of climatic conditions suitable for the pathogen to infect plant tissues is quite wide), disease forecasting models are commonly not used to schedule chemical treatments against grey mould on the grapevine. This is possibly owing to the fact that none of the models developed so far took into account the complexity of *B. cinerea* epidemiology. Consequently, treatments are applied at fixed phenological plant stages: full bloom, bunch closure, veraison and before harvesting. However, the full fungicide schedule is normally applied only in the event of high disease pressure, and in most locations with low-medium disease pressure, fewer sprays are carried out. For example, under the environmental conditions of most Italian vineyards, treatment at blossoming is skipped, because no or few infections commonly occur at that stage. Recently a new mechanistic, weather-driven model was developed for predicting the risk of grapevine infection by *B. cinerea* during two infection periods (from the stage ‘inflorescence clearly visible’ to ‘berries groat-size’ and from the stage ‘berry touching’ to ‘berries ripe for harvest’). This model gave very promising results calculating the infection severity in the two periods, correctly classifying the severity of 17 out of 21 epidemics and opening new perspective for using forecasting models to schedule treatments to control grey mould (Gonzalez et al., 2015).

In recent years, the use of microbial biofungicides based on microbial biocontrol agents has increased continuously, because of public concerns regarding the risk of pesticide residues in food and their negative impact on the environment (Fillinger and Elad, 2016). An additional reason to reduce the use of synthetic chemical fungicides against *B. cinerea* is the fast, rapid and relatively easy selection of resistant strains against single-site fungicides in *B. cinerea* populations, caused by continuous use of active ingredients with the same mechanism of action (Fillinger and Elad, 2016; Schnabel, 2016). Microbial biocontrol agents may represent an alternative to these synthetic chemicals; indeed, they normally have multiple mechanisms of action (Vos et al., 2015), which are surmised to prevent or at least significantly slow down the build-up of fungicide-resistant populations. Because of the economic impact of grey mould, several microbial biocontrol agents and non-synthetic chemicals used to combat the disease have been studied (Jacometti et al., 2010). Among the microorganisms, several fungal and bacterial strains have been successfully tested against grey mould on a variety of crops, including the grapevine (Elmer and Reglinski, 2006).

The *Trichoderma* genus has been a valuable source of microbial biocontrol agents for a long time (Vos et al., 2015). *Trichoderma* spp. can be easily isolated from soil, wood and decaying plant material, but they may also be excellent root colonisers (Vinale et al., 2008). *Trichoderma* spp. strains are characterised by multiple mechanisms of action (induction of plant resistance, mycoparasitism, antibiosis and competition for space and nutrients), which may all result in the reduction of plant diseases (Rossi and Pattori, 2009; Vinale et al., 2008; Vos et al., 2015). In addition, *B. cinerea* often penetrates plant tissue through wounds and takes advantage of senescing host tissues to survive and act as an inoculum for infections of berries. Therefore, by colonising these senescing tissues and competing with *B. cinerea*, *Trichoderma* spp. can prevent or reduce grey mould infections (Card et al., 2009). A specific strain, *T. harzianum* T39, was the first biofungicide marketed to combat *B. cinerea* on the grapevine (O’neill et al., 1996).

Aureobasidium pullulans is a widespread and common fungal grapevine epiphyte. Strains of *A. pullulans* were initially developed to control post-harvest diseases, including grey mould (Bencheqroun et al., 2007; Lima et al., 1997; Zhang et al., 2010). However, *A. pullulans* was also shown to be highly effective against grey mould in greenhouse conditions, for example on cucumbers and tomatoes (Dik and Elad, 1999), and in the field on the grapevine (Elmer and Reglinski, 2006). Natural strains of *A. pullulans* present on grapes or in must/wine are good antagonists of *B. cinerea* (Raspor et al., 2010). The main mechanism of action is based on competition with the pathogen for nutrients at the infection site, although hydrolytic enzymes are also produced (Castoria et al., 2001; Di Francesco et al., 2015a). It was recently demonstrated that *A. pullulans* produces volatile organic compounds that can prevent the germination of conidia of several pathogens, including those of *B. cinerea* (Di Francesco et al., 2015b), making the mechanism of action of this biocontrol agent more complex than previously thought or determined (Spadaro and Droby, 2016).

Strains of *Bacillus subtilis*, *Ba. pumilus* and *Ba. amyloliquefaciens* can control *B. cinerea* (Elad et al., 1994; Mari et al., 1996), mainly through the production of antibiotics (Leifert et al., 1995), although induction of resistance has also been reported to occur in several crops (Choudhary and Johri, 2009). Biofungicides based on spore-forming *Bacillus* species have the advantage of a long shelf-life, a wide spectrum of activity and a generally high compatibility with most synthetic chemical fungicides (Emmert and Handelsman, 1999).

One of the main practical constraints in the use of microbial biofungicides is their variable efficacy, which is mainly due to unfavourable environmental conditions impairing their survival in the canopy or the intrinsically lower level of disease control as compared to synthetic chemicals. To partially solve these problems, a mix of two or more microorganisms has been proposed (Sylla et al., 2015). Several studies have been carried out on combinations of two or more microorganisms in one treatment (Guetsky et al., 2002; Sylla et al., 2015; Xu et al., 2011) with differing results. On the other hand, strategies in which different biocontrol agents are applied in sequence throughout the season have received little attention. In contrast to mixtures of different