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Ph.D. in Agro-Food System
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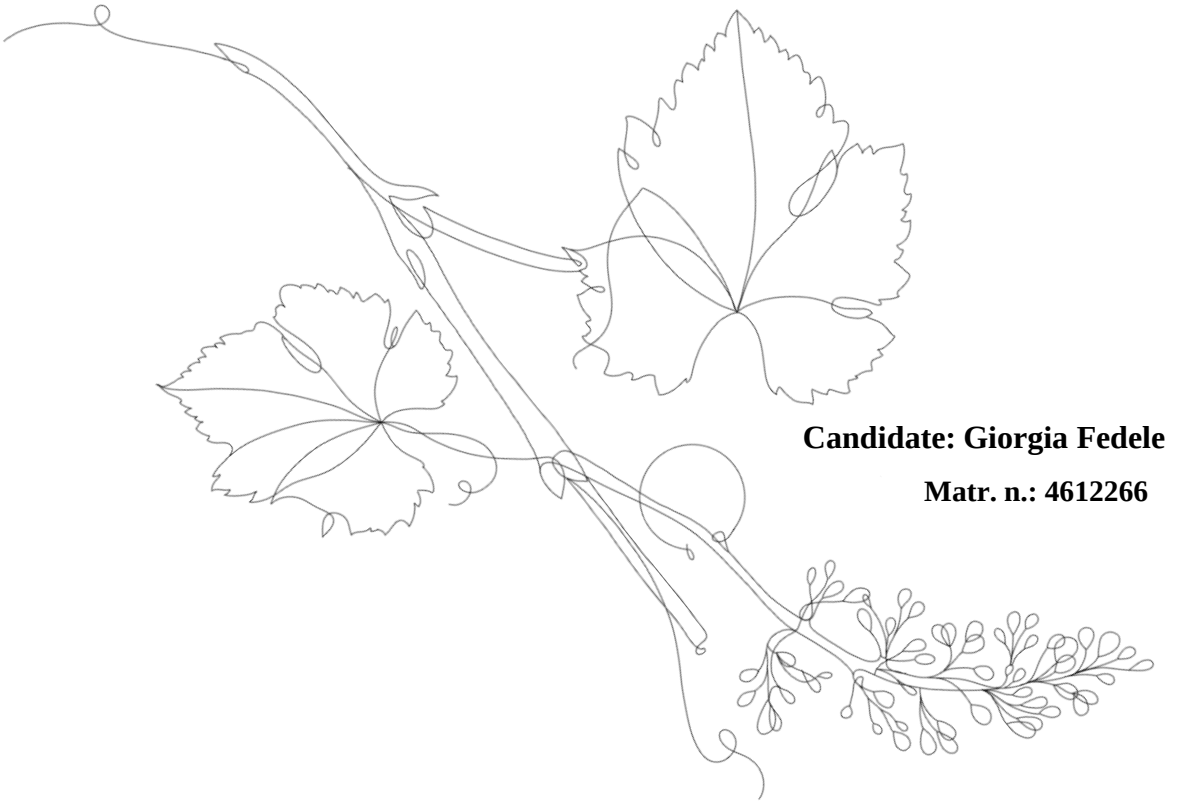
S.S.D.: AGR/12

Coupling botanical epidemiology and mathematical modeling for the control of *Botrytis cinerea* in vineyards



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Academic Year 2018/2019

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INTRODUCTION

Botrytis cinerea Pers.:Fr. is the causal agent of Botrytis bunch rot (BBR), a severe disease in vineyards (Elmer and Michailides 2007; Williamson et al. 2007). The fungus affects all vine organs and thereby reduces both the quantity and quality of the harvested grapes. *Botrytis cinerea* can develop and actively grows as a necrotrophic pathogen and as a saprophyte, affecting at least 580 genera of vascular plants and crop products during cultivation, storage, and transport (Elad et al. 2016). It can occur wherever its host plants are grown, ranging from tropical and subtropical areas to cold temperate zones and involves severe disease management problems all around the world (Elad et al. 2016). In grapevine, loss of crop yield results from damage to inflorescences before flowering, flowers during flowering, young berries at fruit set, and berries during ripening; the latter damage is referred to as bunch rot. Additionally, the fungus can cause early latent infections at flowering time, which damage the fruits after ripening (Jarvis 1977). Quality is reduced because rotted berries have an altered chemical composition that causes undesirable flavours in wine (Steel et al. 2013).

The pathogen

Botrytis cinerea Pers.:Fr. is the anamorphic stage of *Botryotinia fuckeliana* (de Bary) Whetzel (Kirk et al. 2008), and is included in *phylum Ascomycota*, class *Leotiomycetes*, order *Helotiales*, family *Sclerotiniaceae*. The genus *Botrytis* was first described by Pier Antonio Micheli in 1729 and contains more than 30 species (Elad et al. 2016). This genus is closely related to *Sclerotinia*, with the proteins encoded by the genomes of *B. cinerea* and *S. sclerotiorum* displaying 83% identity (Amselem et al. 2011).

In the asexual stage, the mycelium of the species belonging to the genus *Botrytis* produces macroconidia (the *Botrytis* anamorph form), microconidia (spermatia), and sclerotia (Beever and Weeds 2007). In the sexual stage, microconidia may fertilize sclerotia to produce apothecia (the *Botryotinia* teleomorph stage), and ascospores are produced (Beever and Weeds 2007). The connection between the anamorph *B. cinerea* and its teleomorph *B. fuckeliana* (sensed by de Bary, 1866) has been established by Groves and Loveland (1953) and confirmed by Faretra et al. (1988a; 1988b). The *Botrytis*-research community recently decided to retain the asexual name, *Botrytis*, for fungi from this genus, and the teleomorph name should be used for the only *Botryotinia* species lacking a *Botrytis* equivalent (Johnston et al. 2014a).

Botrytis cinerea is heterothallic (Groves and Loveland 1953); the mating is controlled by a single locus (*MAT1*) with two idiomorphs: *MAT1-1* and *MAT1-2*

(Faretra et al. 1988a; 1988b). Few *B. cinerea* isolates fertile with both *MAT1-1* and *MAT1-2* strains and often auto-fertile, referred to as pseudo-homothallic (*MAT1-1/2*) or 'dual-mater' (Amselem et al. 2011; Faretra and Grindle 1992). Heterokaryosis has traditionally been considered very important in phenotypic variability of the fungus (Büttner et al. 1994; Hansen and Smith 1932). In particular, it is the leading cause of secondary homothallism of field and monoconidial isolates of *B. cinerea*, since single multinucleate conidia may contain nuclei carrying opposite idiomorphs (Faretra et al. 1988b).

Some studies showed that *B. cinerea* exhibits great phenotypic diversity (Chardonnet et al. 2000; Martinez et al. 2003; Yourman et al. 2001). Phenotypic diversity is caused by not only heterokaryosis or genes present in chromosomes but also by the presence of variety extrachromosomal genetic elements, such as mitochondrial DNA (De Miccolis Angelini et al. 2004; Yin et al. 2012), plasmids (Hiratsuka et al. 1987) and mycoviruses. These elements are likely transmitted via conidia, while transmission via ascospore progeny may, in some cases, be limited or occurs only via the maternal parent (Beever and Weeds 2007).

Transposable elements (TEs) also contribute to phenotypic variability (Levis et al. 1997; McDonald 1993). In general terms, TEs can be divided into class I retroelements (LTRs) or class II DNA transposons (MITEs, TIRs) (Daboussi 1996; Kidwell and Lisch 2001). Two types of strains, named *transposa* and *vacuma*, have been distinguished based on two transposons, *Boty* and *Flipper* (Giraud et al. 1999). The presence/absence of these two elements has been used to describe four transposon types in populations: *vacuma* (strains with neither of these elements), *transposa* (strains with both elements), *Boty* and *Flipper* (strains with one or other of the two elements) (Muñoz et al. 2002; Albertini et al. 2002; De Miccolis Angelini et al. 2004; Fournier et al. 2005; Isenegger et al. 2008; Martinez et al. 2008; Esterio et al. 2011). Frequencies of strains belonging to the different transposon types in the fungal population in vineyards were highly dependent on geographic location (Muñoz et al. 2002), isolation year (Váczy et al. 2008), and sampling time over the season (Martinez et al. 2005, 2008). Some studies led to the establishment of strains belonging to *vacuma* as the new species *B. pseudocinerea* (Walker et al. 2011; Fournier et al. 2005). However, this classification has become obsolete, because some *transposa* strains were found in *B. pseudocinerea* (Walker et al. 2011; Johnston et al. 2014b; Fekete et al. 2012).

Life cycle and Epidemiology

Botrytis cinerea has a necrotrophic lifestyle: after infection and death of host tissue, the fungus can survives and sporulates as saprophyte on the necrotic tissue including grape debris and weeds covering the soil (Elmer and Michailides 2007;

Jarvis 1977), or produces long-term survival structures (Holz et al. 2007). In late winter and early spring, conidia can be produced on over-wintering mycelium (Nair et al. 1995; Mundy et al. 2012; Seyb et al. 2000; Jaspers et al. 2013) and sclerotia within grape debris (old rachides, tendrils, mummified berries, dead leaves, and canes) (Elmer and Michailides 2007; Jarvis 1977; Holz et al. 2007; Nair and Nadtotchei 1987). Conidia can also be produced on alternative hosts close to vineyards (Elmer and Michailides 2007).

Botrytis cinerea inoculum, in the form of conidia, is abundantly present in the vineyard throughout the growing season (Rodríguez-Rajo et al. 2010) and can be produced on grape bunch, bunch and leaf trash, and rotted berries under a wide range of environmental conditions (Ciliberti et al. 2016; Mundy et al. 2012; Nair et al. 1995). Mature conidia become airborne with a circadian periodicity, the maximum concentrations in the air occurs at about midday, and this periodicity is correlated positively with increasing temperature and wind velocity and negatively with increasing relative humidity (RH) and the presence of dew (Carisse 2016). Conidia are dispersed by wind, rain, and insects (Holz et al. 2007). When conidia are deposited on plant surfaces, they germinate by producing a germ tube; the germ tube forms an appressorium that penetrates the host surface actively by mechanical and enzymatic activity (Salinas and Verhoeff 1995; Holz 1999; Coertze and Holz 2002). *Botrytis cinerea* is an opportunist pathogen that can also initiate infection by passive penetration through wounds, lesions caused by other pathogens or insect pests, and open stomata (Puche-Planté and Mercier 1983).

Several factors influence germination of conidia. Latorre and Rioja (2002) found that conidial germination occurs from 5 to 30 °C, with no germination at 0°C and optimum at 20°C. Similar results have been obtained by Ciliberti et al. (2015a), which observed a rapidly germination at 20°C and complete conidia germination after 24 h at temperatures between 10 and 30°C, no germination occurs at 40°C. No conidial germination occurs in the absence of free water on the host surface (Brown 1916; Blackman and Welsford 1916; Snow 1949; Williamson et al. 1995). However, relative humidity (RH) >90% can provide the free water needed for germination (Ciliberti 2014; Latorre and Rioja 2002). *Botrytis cinerea* is able to grow at 0°C (Schneider-Orelli 1912; Shiraishi et al. 1970), and optimal temperature for mycelial growth is between 20 and 25°C (Brooks and Coley 1917; Ciliberti 2014; Shiraishi et al. 1970; Jarvis 1977); the growth rate decreases markedly above 25°C (Jarvis 1977). The pathogen needs >0.95 a_w for optimal growth (Ciliberti 2014; Magan and Lacey 1984; Lahlali et al. 2007; Deytieux-Belleau et al. 2009).

In the early season, *B. cinerea* affects young leaves, young succulent shoots, and inflorescences in wet conditions (Jarvis 1977; Elad et al. 2007).

Leaves show a characteristic v-shaped arc of dead brown tissue, often surrounded by a yellow margin extending from the edge of the leaf into the central veins (Jarvis 1977). Shoots show soft brown spots and often break at the nodes revealing brown discoloration of the internal tissues (Jarvis 1977). Young inflorescences develop brown, musty rot patches along the peduncle until the inflorescence is completely rotted.

Grape inflorescences can be affected at any growth stage before flowering or to flowering, but they are more susceptible at flowering, fruit swelling, or “berry groat-size” than at earlier growth stages (Jarvis 1977; Ciliberti et al. 2015a). Flowers are highly susceptible to infection during flowering and when they senesce (Jersch et al. 1989) because of low resveratrol content (Keller et al. 2003) and the abundance of pollen (Ogawa and English 1960). In the early season, infection severity increases after brief exposure to wetness at temperatures near 20°C (Ciliberti et al. 2015a; 2015b; 2016; Latorre and Rioja 2002; Nair and Allen 1993). At 5, 10, and 30°C, low levels of infection occur (Nair and Allen 1993; Broome et al. 1995; Latorre and Rioja 2002).

From flowering to young cluster, conidia germinate by producing a germ tube and infect the flower styles and ovules (pathway I), the stamens or petals (pathway IIa), or the fruit pedicel (pathway IIb) (Elmer and Michailides 2007). In Pathway I, the infection of the stylar tissue is followed by slow systemic hyphal growth into the ovule where *B. cinerea* enters in a latent phase (McClellan and Hewitt 1973; Nair and Parker 1985). In Pathway IIa, after the infection of stamens, the pathogen basipetally grows into the receptacle and vascular tissue of berries, where the fungus enters in the latent phase (Pezet and Pont 1986; Viret et al. 2004). In addition, in Pathway IIb, the infection of the fruit pedicel is followed by latent infections (Elmer and Michailides 2007). After veraison, under suitable environmental conditions, latent infection of young berries may become visible as rotted berries and may contribute to final disease severity, but the contribution of latent infections to final disease remains unclear (Holz et al. 2007; Keller et al. 2003; McClellan and Hewitt 1973).

During the flowering stage, the pathogen also saprophytically colonizes the bunch trash (the dead stamens, aborted flowers, aborted berries, calyptras, and tendrils) retained within the developing bunches and then occurring conidial germination and extensive colonization of floral debris in grape bunches (pathway III, Elmer and Michailides 2007). Blighted inflorescence parts and floral debris have been considered a major source of inoculum within developing bunches (Nair and Parker 1985; Calvo-Garrido et al. 2014b) and have been correlated with the incidence of infected berries at harvest (McClellan and Hewitt 1973; Seyb et al. 2000; Calvo-Garrido et al. 2014b). Besides, the mycelium colonizing the bunch

trash can produce conidia under favorable conditions, resulting in a source of inoculum for new infections of the ripening berries (pathway IV, conidial accumulation within the developing bunch; Elmer and Michailides 2007).

After veraison, a classical pre-harvest polycyclic epidemic can develop under favorable weather conditions; rot develops, and a new crop of conidia are dispersed to new infection sites and cause infections of ripening berries (pathway Va; Elmer and Michailides 2007). In addition to conidial infection, ripening berries can be infected through contact with the aerial mycelium produced on adjacent moldy berries (pathway Vb, berry-to-berry infection; González-Domínguez et al. 2015).

From veraison to ripening, the berry infection rate is highest at temperatures between 15 and 25°C and increases with hours of wetness or high relative humidity (Broome et al. 1995; Ciliberti et al. 2015b; Latorre and Rioja 2002; Nair et al. 1988; Nair and Allen 1993). The appearance of symptoms is also promoted by the increased susceptibility of berries approaching maturity; this increase has been associated with structural and biochemical changing during this period (Deytieux-Belleau et al. 2009; Hills et al. 1981; Kretschmer et al. 2007; Mundy and Beresford 2007). Early symptoms of infection in ripening berries are the formation of small circular water-soaked (Jarvis 1977). When rubbed, the skin over these areas cracks and slips freely, revealing the inner pulp; this is known as the “slippery-skin” stage (Jarvis 1977). Usually, the skin ruptures in the center of the rotted area, and conidiophores with conidia develop in this broken area under high relative humidity or moisture (Jarvis 1977). In compact bunches, the rot can spread rapidly from berry to berry until entire bunches are rotted and covered with grey mold (Jarvis 1977).

Characters of the cultivar and cultural practices may influence the compactness of bunches and density of vegetation, increasing the wetness duration within bunches and consequently, the disease incidence (Jarvis 1977; Mlikota Gabler et al. 2003; Vail and Marois 1991). Excessive nitrogen fertilization leads to excessive vigor of clusters with high bunch compactness and cuticle thinning (Mundy and Beresford 2007). Calcium deficiency also influences the skin thickness and susceptibility to *B. cinerea* (Elad et al. 1992). Moreover, wounds caused by different factors increase the infection of berries (Jarvis 1977; Nair et al. 1988; Mundy and Beresford 2007).

Disease control

BBR is commonly managed by routine applications of fungicides at four specific grape growth stages (GS): A, end of flowering (GS69 of Lorenz et al. 1995); B, pre-bunch closure (GS77); C, veraison (GS83); and D, before harvest (before

GS89) (Bulit et al. 1970; Nair and Allen 1993; Broome et al. 1995; Elmer and Michailides 2007; Deytieux-Belleau et al. 2009). This routine application of fungicides has become increasingly unacceptable for the high risk and effects of chemical use on human health and the environment (Alavanja et al. 2004; Epstein 2014). Moreover, *B. cinerea* populations frequently develop resistance to fungicides (Leroux 2007), which is difficult to avoid with the current resistance-management strategies (Fernández-Ortuño et al. 2016). Consequently, alternative approaches for systematic disease suppression are urgently needed. This goal can be achieved following an integrated pest-management framework, either by applying fungicides only when really needed, thus eliminating unnecessary sprays, or by integrating chemical and non-chemical measures, agronomic practices or applications of biopesticides (i.e., preparations based on living microorganisms and substances of natural origin; Nicot et al. 2016) (Fillinger and Walker 2016). The forecast models or Decision Support Systems (DSS) could also help to achieve this goal (Rossi et al. 2012).

Fungicides. Chemical control is based on the application of synthetic fungicides and constitutes the principal means of efficient and reliable grapevine protection against BBR (Leroux 2007; Fillinger and Walker 2016).

The fungicides mostly used to control BBR target either specifically essential cellular functions (single-site activity), or display multi-site activity, interfering with more than one cellular function (Fillinger and Walker 2016). Conventional synthetic fungicides used for *Botrytis* control are: i) succinate dehydrogenase inhibitors, which act by blocking fungal respiration (e.g., boscalid, penthiopyrad, fluopyram, isofetamid, and adepidyn); ii) methionine fungi biosynthesis interferers (e.g., cyprodinl and pyrimethanil); iii) quinone outside inhibitors (e.g., pyraclostrobin); and iv) sterol biosynthesis inhibitors (e.g., fludioxonil and fenhexamid) (Avenot and Michailides 2010; Bardas et al. 2008; Grabke et al. 2013). The Fungicide Resistance Action Committee have classified the risk of resistance of these fungicides by their modes of action (FRAC, 2019); moreover, *B. cinerea* is classified as a high-risk pathogen for acquired resistance development (FRAC, 2019), due to its broad variability and adaptability. Fungicide resistance has been experienced with almost all fungicides used against *B. cinerea* and in numerous countries (Asadollahi et al. 2013; Fillinger and Walker 2016; Fernández-Ortuño et al. 2016; Hahn 2014). Resistance to multi-site fungicides has been observed only in a few cases (Leroux 2007).

For these reasons, fungicides should be applied by following some anti-resistance strategies aiming to reduce the development of acquired resistance in fungal populations. These strategies should combine the biological risk (inherent to the fungus' life traits), the fungicide risk (inherent to the fungicide's mode of

action), and the agronomic risk (reflecting cultural practices and the intensity of fungicide use) (Kuck and Russell 2006). Anti-resistance strategies include: i) the avoidance of repeated applications of fungicides with the same modes of action; ii) the use of mixtures or alternate non-cross-resistant fungicides in situations requiring multiple spray applications; iii) the use of fungicides with high risk of resistance only in the critical part of the season (only when strictly necessary), and if mixture or alternation is not possible; iv) the use of fungicides as recommended on the label without reduced doses; and v) the use of agronomic practices to reduce the risk of disease. However, the current anti-resistance management strategies have not been able to prevent the independent development of resistance to site-specific fungicides (Fernández-Ortuño et al. 2015). In this sense, the last findings encourage a limited use of fungicides and the alternation of active ingredients at full dose with different modes of action, more than the mixture strategy (Fillinger and Walker 2016).

Biopesticide. In recent years, researchers have been increasingly exploring alternatives to chemical control, including the use of living microorganisms (referred to as biocontrol agents or "BCA") and substances of natural origin such as microbials, botanicals, minerals and organic compounds (Nicot et al. 2016). A wide range of different biopesticides have been reported to exhibit inhibitory activity against *B. cinerea* in laboratory and greenhouse trials, but only a few have shown consistent field performance, and even fewer have been commercialized (Nicot 2011; Elmer and Reglinski 2006).

BCAs include control products for *B. cinerea* with active ingredients ranging from yeasts, fungi, and bacteria. These microorganisms may suppress the pathogen via competition, antibiosis, and/or parasitism (Elmer and Reglinski 2006; Elad and Stewart 2007; Haidar et al. 2016). In vineyards, the most common biocontrol agents applied are: *Trichoderma* spp. (O'Neill et al. 1996; Elad and Stewart 2007; Pertot et al. 2017; Longa et al. 2009); *Ulocladium* spp. (Reglinski et al. 2005; Elmer et al. 2005; Schoene 2002); *Aureobasidium* spp. (Elad and Stewart 2007; Pertot et al. 2017; Elmer and Reglinski 2006); *Candida* spp. (Calvo-Garrido et al. 2014a; 2014c; Carbó et al. 2017); and *Bacillus* spp. (Thomidis et al. 2016; Pertot et al. 2017; Jacometti et al. 2010).

Plant extracts and other plant-based compounds such as essential oils have been tested for their efficacy in the management of a wide range of fungal diseases in plants (Abbey et al. 2018; Amini et al. 2012; Bhagwat and Datar 2014; De Corato et al. 2017). In recent years, several essential oils have been reported to have high antifungal activities and their acquired popularity in the agricultural sector is due to their volatility, ecological, and biodegradable properties (Abbey et al. 2018). Essential oils naturally contain bioactive compounds that can effectively manage

B. cinerea in vitro, but only a few inhibit *B. cinerea* infection in grape bunches and in leaves *in planta* (Walter et al. 2001; Jacometti et al. 2010; Rotolo et al. 2018). Although the specific modes of action of these essential oils are still unclear, it is believed that they act against the cytoplasmic cell membranes of microorganisms (Diao et al. 2014).

In addition, mineral oils can be effective at suppressing *B. cinerea* (Jacometti et al. 2010). Applications of these oils are effective at low dosages because of their excellent spreading and sticking properties and, if highly refined, are quite disease-specific (Jacometti et al. 2010).

Moreover, various biotic and abiotic compounds can elicit a plant defense response that can be highly effective at suppressing *B. cinerea* in grapes (Elmer and Reglinski 2006; Reglinski et al. 2005). These compounds include plant hormones, abiotic stimulants, plant and microbial extracts, and microbes (Jacometti et al. 2010).

Agronomic practices. Agronomic practices also contribute to reducing the incidence and severity of *B. cin*erea (Gubler et al. 1988; English 1989; Valdés-Gómez et al. 2008). Agronomic practices include canopy management (e.g., leaf removal, bunch trash removal, leaf plucking, shoot thinning, and pruning), soil and weeds management, reduction of nitrogen fertilization, and irrigation. All these practices influence the microclimate within the canopy and promote unfavorable conditions for disease development, as previously described. Moreover, leaf removal can contribute to reduce the compactness of bunch or increase the thickness of berry skin when applied in specific phenological stages (Fregoni 1998). Similar effects on the thickness of the skin may be due to foliar application of calcium fertilizers (Elad et al. 1992). Lastly, the control of grape berry moth (*Lobesia botrana*) and powdery mildew (*Erysiphe necator*) is essential to reduce the damage of berry skin (Jarvis 1977; Elad et al. 2007).

Empirical rules, prediction models and an expert system for BBR control

Use of prediction models embedded in warning or decision support systems is an efficient way to make decisions on whether and when it is necessary to control diseases (Madden et al. 2007; Rossi et al. 2012). Only a few works have been carried out to model the development of *B. cinerea* in vineyards.

An empirical rule was proposed by Baldacci et al. (1962) and by Bulit et al. (1970). Baldacci et al. (1962) suggested to applying fungicides at three determined grape growth stages: (A) end of flowering; (B) pre-bunch closure; and (C) veraison. Treatments were proposed according to the susceptibility of grapevine at these growth stages after several observations in a vineyard of North Italy (Baldacci et al. 1962). Agulhon (1969) improved the method suggesting a fourth treatment (D)

to perform 3-4 weeks before harvest. Then, this method has been widely tested and commonly known as the “phenological method” in France (Agulhon 1969; 1973; Lafon et al. 1972), Italy (Bisiach 1975; 1978a; 1978b), Germany (Gartel 1977) and Swiss (Bolay and Rochaix 1975).

At the same time, Bulit et al. (1970) formulated the “two fifteens rule”: an infection by *B. cinerea* may occur when the grapes remain wet for 15 consecutive hours at a temperature of at least 15 °C. This rule was developed based on field experiments consisted in preventative applications of fungicide during the season, recording of weather data (rain, temperature, and hours of wetness), assessment of conidia in the air and evaluation of BBR symptoms. This method has been tested in France (Lafon et al. 1972) and Italy (Bisiach et al. 1978a; Egger et al. 1994). This method allows to reduce the number of treatments in years little rainy (Bisiach et al. 1978) but not ensure constant results (Egger et al. 1994). In the following decades, four empirical models were developed for BBR (Strizyk 1985; Nair and Allen 1993; Broome et al. 1995; Rodríguez-Rajo et al. 2010).

The EPI-Botrytis (Etat Potentiel Infection - Botrytis) model of Strizyk (1985) is an empirical model that predicts infection risk of BBR according to the susceptibility of the cultivars, the phenological stages, the quantity of airborne conidia, and the weather conditions. The model daily updates the EPI index according to wetness periods or relative humidity, temperature, and an empirical index of the number of conidia in the air considering the hours of day and phenological stages. The EPI index is an estimation of the quantity of bunches infected at harvest by BBR (Molot 1987). The model suggests if a fungicide treatment is required at the four grape growth stages considered critical in standard anti-Botrytis strategies (Molot 1987): end of flowering (A), bunch closure (B), veraison (C), and three weeks before harvest (D). A treatment is required when EPI index exceeds a specific threshold for each critical stage. The model was extensively validated in France and Italy (Molot et al. 1983; 1987; Egger et al. 1994; Brunelli and Cortesi 1990). The EPI-*Botrytis* model described with sufficient precision the development phases of BBR, with underestimation of infection risk at harvest (Molot 1987; Egger et al. 1994; Brunelli and Cortesi 1990; Molot et al. 1983). This model permitted a good protection of vineyards, reducing the number of fungicide treatments according to the phenological method and ensured more constant results than the “two fifteens rule” (Molot et al. 1983; 1987; Egger et al. 1994).

Nair and Allen (1993) developed a simple model to predict the percentage of infection of *B. cinerea* on grape flowers and berries as a function of wetness duration and temperature. Grape flowers and mature berries were used in laboratory experiments to collect the data needed to develop the model. The model

was not validated in the field, and there are no indications on how to use the model for deciding when a fungicide application is necessary.

Broome et al. (1995) developed a model to predict the incidence of BBR infection on mature berries as a function of wetness duration and temperature. To develop the model laboratory data were used and a multiple regression model was developed to calculate an infection index as a function of wetness duration and temperature. Broome et al. (1995) found that the infection rate increased with increasing wetness duration. The model was linked to an action threshold, and in this system, the fungicide applications were based on the level of risk that a grower is willing to accept. The model was programmed into an automated weather station (Envirocaster) and validated for two years on Chilean table grapes; fungicide applications based on the model allowed growers to reduce the use of fungicides by approximately 50% with equivalent disease control.

A model for the prediction of airborne *B. cinerea* conidial concentrations was developed in Spain (Rodríguez-Rajo et al. 2010). A phenological and aerobiological survey was carried out from 2004 to 2008 on three grape varieties. Spore samplings were obtained by a volumetric spore trap located in the vineyards and related to weather variables trough an ARIMA (Autoregressive Integrated Model of Running Mean) time-series model. The model based on the dew-point occurring two days earlier was able to predict the *B. cinerea* airborne conidial concentrations with a prediction horizon of 24 hours. Validation was carried out with data of spore counts in 2008; the model predictions matched observed spore counts in most cases. The model is proposed to be used to apply fungicides when the concentration of spores in the air is high.

In the late 1990s an expert system was developed in Australia as a set of "if-then" rules organized in a decision tree made up of three main sections: i) "fungicide coverage" (i.e., the residual presence or absence of a previous treatment); ii) the "economic threshold" (e.g., grapevines for premium quality or bulk wine); and iii) "disease risk" (e.g., due to a combination of different factors such as injury, growth stage, susceptibility, infections and symptoms) (Ellison et al. 1998a). The knowledge on *B. cinerea* population dynamics were used to estimate the disease risk (in a zero to one scale) according to: i) growth stage of grapevines; occurrence of infection by ii) conidia or iii) mycelium; iv) lesions on berries; v) presence of disease symptoms; and vi) cultivar susceptibility. The expert system uses two economic thresholds of damage, one for low-quality wine and one for high-quality wine. The "fungicides" component considers the duration of plant protection after the application of fungicides. The expert system produces a response to establish if applications of fungicides are necessary; a fungicide application is required when the risk of disease exceeds the economic thresholds

of damage, and the crop is not already protected against BBR. The expert system was validated from 1990 to 1994 in Australia (Ellison et al. 1998b).

A Decision Support System (DSS) called the Botrytis Decision Support (BDS) was developed by Beresford et al. (2012) in Australia and New Zealand. The BDS model captures the main features of *B. cinerea* biology and simulates the effects of risk factors and control options on the development of BBR epidemics. The BDS system consists of an early-season model and a late-season model. The early-season model assesses the effects of weather on Botrytis risk by accessing vineyard weather stations. In particular, the Botrytis risk index is estimated using an algorithm, called the 'Bacchus model', which uses temperature and wetness data to determine the daily risk of infection by *B. cinerea* (Kim et al. 2007). The late-season Botrytis risk model predicts BBR incidence according to berry sugar accumulation (°Brix) between veraison and harvest. Results from vineyard studies were used to calibrate the BDS model, and subsequently, it was turned into an online Botrytis management tool. The BDS system is being implemented for the wine industry of Australia and New Zealand.

Recently, a new, mechanistic model for *B. cinerea* on the grapevine was developed González-Domínguez et al. (2015). The model was developed using the results of recent publications that investigated the effect of environmental conditions on the biology and epidemiology of *B. cinerea* isolates belonging to different transposon genotypes (Ciliberti et al. 2015a; 2015b; 2016). This model accounts for the full complexity of the *B. cinerea* life cycle and of Botrytis bunch rot epidemiology, and considers two infection periods. In the first infection period (between "inflorescences clearly visible" and "berries groat-sized, bunches begin to hang"), the model calculates a daily infection risk (RIS1) for infections by conidia on inflorescences and young clusters (pathways I and II of Elmer and Michailides 2007). In the second infection period (between "majority of berries touching" and "berries are ripe for harvest"), the model calculates the daily infection risk on ripening clusters for infections caused by conidia (pathway Va of Elmer and Michailides 2007; RIS2) and for berry-to-berry infection by aerial mycelium (pathway Vb of González-Domínguez et al. 2015; RIS3). The model uses the vineyard's weather data and vine growth stages to predict, on any day of the vine-growing season, the epidemic group (severe, intermediate, or mild) at harvest (González-Domínguez et al. 2015). The model was evaluated with data collected from 21 vineyards in Italy and France and between 2009 and 2014; according to a discriminant function analysis (DFA), the model correctly classified 81% of the epidemics (González-Domínguez et al. 2015). This model is currently integrated into a Decision Support System (DSS) called vite.net (Rossi et al. 2012).

Objectives of the Thesis

In the last years, the control of BBR based on calendar sprays of fungicides has been considered no more sustainable because of its important limitations. First, the treatments are preventive and do not take into account the real risk of infection, the treatments are sometimes unnecessary. Second, this method can increase the probability that *B. cinerea* develops resistance to fungicides. Finally, public concerns about the possible effects of chemicals on human health and environmental pollution require that fungicides not be applied when unnecessary. Thus, the sustainable control of BBR should include: i) the integration of the epidemiology knowledge of the disease with the fungicide applications strategies; ii) the applications of fungicides only when necessary considering the real risk of BBR development; and iii) the use of alternative products, like biological control agents (BCAs) and botanicals to control BBR. Following these aims, the research developed in this Thesis includes:

A multi-treatment (or network) meta-analysis to compare different strategies of BBR control, based on combinations of 1, 2, 3, or 4 sprays applied in A, B, C, and/or D (**Chapter 2**);

The analysis of the interactions among fungicide treatments applied at different timings for the control of BBR in vineyards (**Chapter 3**);

The optimization and validation of a hydrolysis probe-based qPCR assay for the quantification of *B. cinerea* DNA in bunch trash (**Chapter 4**);

The evaluation of the effects of different products (fungicides, biological control agents, and botanicals) applied at different timings on bunch trash colonization on and sporulation of *B. cinerea* (**Chapter 5**);

The validation of the weather-driven mechanistic model developed by González-Domínguez et al. (2015) and the evaluation of its ability to account for latent infections (**Chapter 6**).

A systematic literature review to study the effect of the environmental conditions on the fitness and efficacy of microorganisms for biocontrol of *B. cinerea* (**Chapter 7**);

The further development of a model for biocontrol proposed by Jeger et al. (2009) by including i) the effect of environmental conditions on the pathogen-BCA interactions and ii) the dynamic of host growth and senescence (**Chapter 8**).

Finally, how the findings rising from the present Thesis can be used to propose new managements of BBR in vineyards are discussed in the Conclusions (**Chapter 9**).

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Chapter 2

Chapter 2

A network meta-analysis provides new insight into fungicide scheduling for the control of *Botrytis cinerea* in vineyards[1]

Abstract

Control of Botrytis bunch rot (BBR) is currently based on the application of fungicides at four timings corresponding to specific growth stages of vines: end of flowering (A), pre-bunch closure (B), veraison (C) and before harvest (D). The current research provides a network meta-analysis of 116 studies conducted between 1963 and 2016 in nine countries, in which 14 strategies (based on combinations of 1, 2, 3, or 4 sprays applied in A, B, C, and/or D) were compared.

When a 1-spray strategy was applied, BBR control was more effective with sprays applied in A, C, or D than B. With a 2-spray strategy, strategy AC provided similar control as strategy BC; strategy CD also provided good control. For a 3-spray strategy, the best disease control was consistently obtained with strategy ACD. Four sprays strategy ABCD provided the best control but often involved needless sprays so that the routine application of four sprays is not justified.

Spraying at timing A seems to be very important for achieving efficient and flexible disease control. Flexibility is reduced by spraying at timing B rather than A.

[1] González-Domínguez E, Fedele G, Caffi T, Delière L, Sauris P, Gramaje D, Ramos-Sáez de Ojer JL, Díaz-Losada E, Díez-Navajas AM, Bengoa P, Rossi V, 2019. Pest Management Science 75:324-332.

Introduction

The fungus *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) infects more than 200 plant species and causes among the most important plant diseases worldwide (Elad et al. 2007). On grapevines, *B. cinerea* causes the devastating disease called Botrytis bunch rot (BBR) (Elmer and Michailides 2007), which can affect all of the herbaceous organs of the vines; damage to ripening berries is especially serious, leading to severe losses in yield and reductions in wine quality (Steel et al. 2013).

The biology of *B. cinerea* and its epidemiology on vine crops have been studied in detail (Elmer and Michailides 2007; Ciliberti et al. 2015a; 2015b; 2016; Deytieux-Belleau et al. 2009), and multiple infection pathways have been identified that occur in two periods: from flowering to young cluster development, and after veraison. In the early season, *B. cinerea* infects inflorescences and young berries, resulting in (i) inflorescence and blossom blight, (ii) latent infections of berries, and (iii) saprophytic colonisation of grape bunch trash (Ciliberti et al. 2015a). After veraison, latent infections may become visible as rotted berries, and the colonized bunch trash may serve as a source of inoculum inside the bunches. In addition to conidial infection, ripening berries can be infected through contact with the aerial mycelium produced on adjacent infected berries (berry-to-berry infection) (González-Domínguez et al. 2015). The susceptibility of berries from veraison to ripening increases according to a sigmoid curve (Deytieux-Belleau et al. 2009; Kretschmer et al. 2007). Modifications of the berry cuticle also make cracks more likely, and the wounded berries can be easily infected (Nair et al. 1988).

The complexity of the life cycle of *B. cinerea* has caused growers to rely heavily on routine applications of fungicides at four specific grape growth stages: A, end of flowering (growth stage 69 of Lorenz et al. 1994); B, pre-bunch closure (growth stage 77); C, veraison (growth stage 83); and D, before harvest (before growth stage 89). This calendar schedule of applications, sometimes called the “phenological method”, was conceived based on the experiments in the 1960s (Agulhon 1969; Baldacci et al. 1962; Bulit et al. 1970). Baldacci et al. (1962) proposed 3 sprays: at the end of flowering, to reduce flower infections and infestation of floral debris; at pre-closure of bunches, as the last chance to disinfest the trash inside the bunch; and at veraison because of the increase in susceptibility of bunches from this period until harvest. Agulhon (1969) improved the method suggesting that a fourth treatment can be applied 3 to 4 weeks before harvest.

The phenological method is easy to follow and provides good protection against BBR (Agulhon 1973; Lafon et al. 1972; Bisiach et al. 1978b). However, the method has important limitations. First, because the treatments are preventive and do not take into account the real risk of infection, the treatments are sometimes

unnecessary. Second, the phenological method can increase the probability that *B. cinerea* develops resistance to botryticides (Leroux 2007). Finally, public concerns about the possible effects of chemicals on human health (Alavanja et al. 2004) and environmental pollution (Epstein 2014) require that fungicides not be applied when unnecessary.

In response to these limitations in the phenological method, researchers have studied the possibility of reducing the number of fungicide applications by identifying the key timings in which fungicides should be recommended. In Europe, these studies have been performed in France (Agulhon 1969; 1973; Lafon et al. 1972), Italy (Bisiach et al. 1978a; Carniel et al. 1980; Flori et al. 1978), Germany (Gartel 1977), Spain (Pérez-Marín 1998), and Switzerland (Bolay and Rochaix 1975). The findings have resulted in varying and sometimes conflicting recommendations for BBR management.

There is therefore a need to assess the effectiveness and consistency of the different management strategies for BBR control. To our knowledge, a quantitative review of multiple studies on different control strategies has not been published. An excellent tool for integrating and interpreting multiple individual studies is meta-analysis (Madden and Paul 2001).

In this work, a multi-treatment (or network) meta-analysis was used to integrate the results of different strategies for BBR control. Network meta-analysis allows direct comparisons of all the strategies to each other and takes into account all of the correlations (Madden et al. 2016). This multi-treatment analysis can also use a large number of individual studies, because it does not require that all of the studies include all of the treatments to be compared. The meta-analysis reported here was preceded by a systematic review of peer- and non-peer-reviewed studies; unpublished data from studies developed or collected by some of the co-authors were also included.

Materials and methods

Database of studies on Botrytis bunch rot control

A database concerning studies of BBR control was assembled from the following sources: (i) JCR (Journal Citation Reports)-indexed journals, (ii) non-JCR journals, and (iii) experimental reports. For (i) and (ii), a structured search on the Web Of Science was carried out using the following search string: (“Botrytis” OR “mould”) AND “grapevine” AND “control”. For (ii), additional searches were performed in the following journals: *Giornate Fitopatologiche*, *EPPO Bulletin*, and *Phytoma* (French and Spanish version), which are not included in the Web Of Science. For (iii), unpublished experiments were considered that were conducted by the co-authors of this report or that were collected from the archives of their institutions. To be

included in the database, an experiment had to meet the following criteria: the experiment included disease severity assessment (*X*); the experiment had a suitable experimental design with at least 3 replicates and an untreated control (NT); and the experiment evaluated at least one fungicide treatment that was applied at timings A, B, C, or D, or at several timings, e.g., strategy AB. For source (i), 22 studies were selected from 5 papers published in *Food Additives & Contaminants*, *Journal of Environmental Science and Health*, *American Journal of Enology and Viticulture*, *European Journal of Plant Pathology*, *Phytopathologia Mediterranea* and *Plant Pathology*. For source (ii), 62 studies were selected from 22 papers published in *Giornate Fitopatologiche*, *Vitis*, *EPPO Bulletin*, *New Zealand Plant Protection*, and *Scientific Papers*. For source (iii), 32 studies were considered from experiments in France (Bordeaux), Spain (Logroño, Ourense, Fraisoro, Laguardia, and Zalla), and Italy (Piacenza, Ravenna, and Cormons). In total, 116 studies were included, and these were conducted between 1963 and 2016 in Australia, France, Italy, Luxemburg, New Zealand, Spain, Romania, Switzerland, and USA. Most studies were conducted with a randomized complete block design, with 4 replicate blocks. The vine variety, the fungicide/s used (active ingredient/s), and application timing varied among studies.

Fourteen treatment strategies were evaluated and were grouped into four types: one spray per season (applied at timings A, B, C, or D); two sprays per season (strategies AB, AC, BC, BD, or CD); three sprays per season (strategies ABC, ACD, ABD, or BCD); or four sprays per season (strategy ABCD). Strategy AD was excluded because it was assessed in only one study. To increase the number of studies for each strategy, a disease severity value (*X*) was calculated from other strategies included in the same experiment when possible. For example, if disease severities were available for strategies ABC and BC (X_{ABC} and X_{BC}, respectively) in one experiment, then the disease severity of strategy A in that experiment was calculated as $X_A = X_{ABC} - X_{BC}$.

Meta-analysis

Effect of fungicide treatments. A network meta-analysis was conducted to evaluate the effect of the different treatment strategies in reducing disease severity compared to the non-treated control (Machado et al. 2017; Paul et al. 2008). For each study and treatment (included the non-treated control), disease severity data were extracted from the publication/report and used to conduct the analysis. A detailed explanation of the procedure is provided in the supplementary material (Analysis explanation). Briefly, the meta-analysis was conducted with the software R (v 3.4.0; package 'metafor'; CoreTeam R 2017; Viechtbauer 2010) by using a multivariate random effects model. Assumptions of residual heterogeneity and

consistency were assessed (Donegan et al. 2013). For heterogeneity, residual heterogeneity (Q_E) was tested and I^2 statistic calculated (Higgins and Thompson 2002; Jackson et al. 2012). The Q_E test evaluates whether the variability in the observed effect that is not accounted by the fungicide treatment strategy is larger than one would expect based on sampling variability only; I^2 was calculated for each treatment and indicates the proportion of total variation in the estimates of treatment effect that is due to heterogeneity between studies. An I^2 of 0% indicates that all of the variability in the estimated effect is due to sampling error within trials, and that none is due to heterogeneity. An I^2 value near 100%, in contrast, indicates that most of the observed variance is due not to sampling error but to variance between studies (Higgins and Thompson 2002). For consistency, the hypothesis that the treatment effect from direct evidence is consistent with the treatment effect from indirect evidence (i.e., calculated cases) was tested (Donegan et al. 2013).

Treatment effect is presented as *L*, the difference (in the log of the severity mean) for each treatment (T) relative to the untreated control (NT) in the form $L_T=\ln(X_T)-\ln(X_{NT})$; the log severity of treatments was used instead of *X* because its distribution is closer to the normal one, as requested by the analysis. Therefore, negative values of *L* indicate that BBR severity was lower in the treated plot than in the NT control, i.e., that the treatment reduced the disease severity compared to the untreated control. Standard errors, confidence intervals, and significant statistics were calculated as described in the supplementary material (Analysis explanation). A Wald-type test statistic was used to determine whether the treatment effects *L* were significantly different from zero, i.e., whether the disease severity in the treated plots $\ln(X_T)$ differed from that in the untreated plots $\ln(X_{NT})$. The percentage of disease reduction relative to the control was also estimated (Ngugi et al. 2011; Paul et al. 2008).

Differences between pairwise combinations. Differences between treatment strategies were tested for all pairwise treatment combinations (i.e., the 14 strategies of 1, 2, 3, or 4 sprays). In total, 98 pairwise combinations were tested by a contrast analysis between the values of *L*.

To assess the across-studies variability, the frequency of studies was determined when (i) X_1 was significantly higher than X_2, (ii) X_2 was significantly higher than X_1, and (iii) no significant differences were observed between X_1 and X_2.

Effect of publication type and fungicide class. A multivariate meta-analysis model was also used to evaluate the effect of two categorical variables: (i) publication type, and (ii) fungicide class. For publication type, studies were categorized as no-JCR, JCR, and experimental report (as described above). For fungicide class, a new database was created that excluded the untreated control, and studies were categorized into 18 groups based on the combination of

fungicides used. Fungicides were grouped based on the chemical classes defined by the Fungicide Resistance Action Committee (FRAC; FRAC 2017). The fungicide class E3 (dicarboximides, including chlozolinate, dimethachlone, iprodione, procymidone, and vinclozolin) was used as reference in the meta-analysis because the fungicides in this class were used, alone or in combination, in 69 of the 116 studies. E3 fungicides have been extensively applied against *B. cinerea* worldwide (Williamson et al. 2007).

A separate analysis was performed for each of the two categorical variables. The interaction between the treatment strategy and these two factors was not evaluated because of the complexity of the models obtained (45 interactions for publication type, and 111 interactions for fungicide class) and because of the low number of cases for some of these interactions.

Results

Database overview

BBR severity in the untreated plots of the 116 studies ranged from 0.1 to 87.4%, with 90% of the values ranging from 3.9 to 64.8%, indicating that the database included a wide range of epidemics (Fig. 2.1). The average disease severity in the untreated controls was 32.5% (s.e. 2.1%) with some asymmetry (0.55) and a negative kurtosis (-0.65). Disease severity also showed high variability among plots treated with fungicides; this variability generally decreased with increasing number of sprays (from 1 to 4) per season (Fig. 2.1). For example, with the 1 spray in A, 90% of the disease severity values ranged from 0.9 to 54.8%; with the 2 sprays in AC, the values ranged from 1.5 to 43.6%; with 3 sprays in ACD, the values ranged from 1.6 to 26.8%; and with 4 sprays in ABCD, the values ranged from 0.2 to 13.2% (Fig. 2.1).

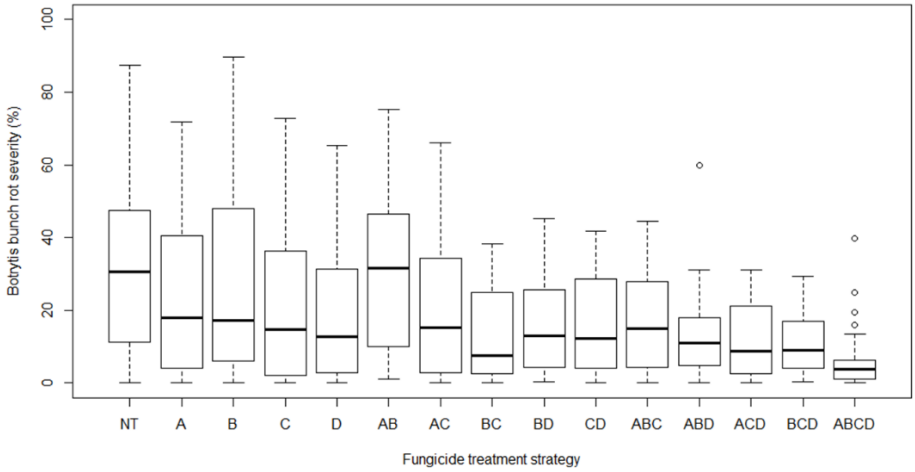

Figure 2.1. Box plots representing the distribution of Botrytis bunch rot severity in different studies in which a fungicide treatment strategy (T) was compared to an untreated control (NT). Strategies are combinations of treatments applied in A (end of flowering, growth stage 69 of Lorenz et al.1994), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and/or D (before harvest, before growth stage 89).

Treatment effects and pairwise comparisons

Both heterogeneity and consistency tests indicated that the results of the meta-analysis can be considered robust. The test for residual heterogeneity rejected the null hypothesis of homogeneity across studies (Q_E= 47672; df = 585; P<0.0001) and the values of I^2 were >80% for all strategies except BC and ABC (Table 2.1). Therefore, the heterogeneity in the estimated *L* values was mainly due to the among-studies variability and not to the sampling errors in each study. Based on the Wald test, no significant interaction between treatment effect and the nature of the case (i.e., if they were calculated) was found (P>0.1), suggesting lack of inconsistency within the dataset used.

The average values of L were significantly <0 for all 14 strategies (i.e., estimated BBR severity was lower in the treated than in the untreated plots; Table 2.1). A value of *L* close to 0 (i.e., the treatment had no/low effect) was estimated for strategy B, whereas *L* values were approximately -0.5 for strategies A, C, and D (Table 2.1). Pairwise comparison by linear contrasts showed that *L* values estimated for strategies A, C, and D were not significantly different from but were lower than the *L* value estimated for strategy B (Table 2.2); therefore, strategies A,

C, and D provided better disease control than strategy B, with the percentage of disease reduction ranging from 36.7% to 41.7% (Fig. 2.2).

Estimated values of *L* were lower compared to the untreated control when 2 sprays were applied instead of 1, except for strategy AB (Table 2.1). The value of *L* for strategy AB was not significantly different than those for strategies A, C, and D, and was higher only than the value for strategy B (P=0.002; Table 2.2). Estimated values of *L* were not significantly different for strategies AC and BC (P=0.082) and were lower than for the single sprays (P<0.05), except when strategy AC was compared with strategy C (P=0.12). When 2 sprays were applied, the lower values of *L* were estimated for strategies BD and CD, which caused with an average disease reduction of 70.5 and 68.4%, respectively (Fig. 2.2). These strategies were not significantly different from each other (P=0.717), and provided better control than all other 1- and 2-spray strategies (P<0.05; Table 2.2).

When 3 sprays were applied, estimated values of *L* were sometimes not significantly different from those values obtained when only 2 sprays were applied. Estimated values of *L* for strategies ABC and ABD were close to -0.7 (48.5 and 53.6% disease reduction, respectively; Table 2.1 and Fig. 2.2). Based on estimated values of *L*, strategies ABC and ABD were only better than strategy AB but were not better than the other 2-spray strategies (Table 2.2). The estimated value of *L* was larger for strategy ABC than for BC, BD, or CD, and the estimated value of *L* was larger for ABD than for BD or CD (P<0.011; Table 2.2). A *L* value of -0.92 was estimated for strategy BCD (60.0% disease reduction), but when compared with 2- and 3-spray strategies, the estimated effect of strategy BCD was significantly lower only than those of AB and ABC (P<0.05; Table 2.2). The lowest value of *L* for the 3-spray strategies was estimated for ACD (L=-1.23; 70.7% disease reduction), which was lower than for all other 3- and 2-spray strategies (P<0.05), except for BD and CD (Table 2.2).

Finally, the value of *L* estimated for the 4-spray strategy ABCD (-1.69) was significantly lower than those for all other strategies (P≤0.007; Tables 2.1 and 2.2). The average disease reduction with strategy ABCD was 81.6% (Fig. 2.2).

The frequency distribution of studies in which the mean severity of one strategy was higher than, equal to, or lower than that of the second strategy revealed substantial variability among individual studies (Fig. 2.3). For instance, when strategy A was compared with D, BBR severity did not significantly differ in 62% of the studies, was significantly lower for D than for A in 35% of the studies, and was significantly lower for A than for D in 3% of the studies (Fig. 2.3). Comparisons concerning the 4-spray strategy ABCD showed that, even though the average (all studies considered) mean severity was significantly higher for ABCD than for all of the other strategies (Table 2.2), the frequency of studies in

which ABCD was not significantly different from that of a second strategy was sometimes high (Fig. 2.3).

No significant differences were observed between the different publication types (P=0.556). In contrast, significant differences were observed for fungicide groups (Table 2.3); the fungicide combinations D1/E2 (anilino-pyrimidines/phenylpyrroles) and E3/MS (dicarboximides/multi-site) significantly (P<0.001) reduced BBR severity compared to the E3 group (dicarboximides), which was used as the reference.

Table 2.1. Effect in the reduction of Botrytis bunch rot severity compared to the untreated control of fourteen fungicide treatment strategies based on 1, 2, 3, or 4 fungicide sprays applied at timings A (end of flowering, growth stage 69 of Lorenz et al.1994), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and/or D (before harvest, before growth stage 89).

Fungicide treatment strategy	K[†]	I^{2}[§]	Estimated effect in disease reduction				
			L[¥]	se of L	95% confidence interval of L		*P*
A	55	93.9	-0.46	0.076	-0.61	-0.31	<0.001
B	44	86.1	-0.25	0.042	-0.33	-0.17	<0.001
C	50	93.9	-0.54	0.060	-0.66	-0.42	<0.001
D	34	89.0	-0.45	0.059	-0.57	-0.34	<0.001
AB	34	97.1	-0.54	0.089	-0.72	-0.37	<0.001
AC	32	93.0	-0.71	0.100	-0.90	-0.51	<0.001
BC	36	66.0	-0.89	0.057	-1.01	-0.78	<0.001
BD	23	90.7	-1.22	0.131	-1.48	-0.96	<0.001
CD	26	94.0	-1.15	0.095	-1.34	-0.97	<0.001
ABC	25	78.1	-0.66	0.075	-0.81	-0.51	<0.001
ABD	26	86.3	-0.77	0.094	-0.95	-0.58	<0.001
ACD	24	84.7	-1.23	0.126	-1.47	-0.98	<0.001
BCD	31	94.8	-0.92	0.102	-1.11	-0.72	<0.001
ABCD	44	81.1	-1.69	0.132	-1.95	-1.43	<0.001

[†] Total number of studies included in the analysis.

[§] I^2 indicates the percentage of total variation in the estimates of treatment effect that was due to heterogeneity between studies. An I^2 value near 100% indicates that most of the observed variance was real, i.e., was not due to sampling error but was due to variance between studies.

[¥] Summary estimated effect for each treatment strategy relative to the untreated control NT, in the form $L_T=\ln(X_T)-\ln(X_{NT})$, where X is the disease severity at harvest.

Table 2.2. Pairwise comparison of the effect in the reduction of Botrytis bunch rot severity compared to the non-treated control for 14 fungicide treatment strategies based on 1, 2, 3, or 4 sprays applied at timings A (end of flowering, growth stage 69 of Lorenz et al.1994), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and/or D (before harvest, before growth stage 89).

Fungicide treatment strategy	**B**	**C**	**D**	**AB**	**AC**	**BC**	**BD**	**CD**	**ABC**	**ABD**	**ACD**	**BCD**	**ABCD**
A	-0.21† (0.020)	0.08 (0.333)	-0.01 (0.980)	0.08 (0.142)	0.25 (<0.001)	0.44 (<0.001)	0.76 (<0.001)	0.69 (<0.001)	0.21 (0.018)	0.31 (0.002)	0.77 (<0.001)	0.46 (0.001)	1.23 (<0.001)
B		0.29 (<0.001)	0.21 (0.002)	0.30 (0.002)	0.46 (<0.001)	0.65 (<0.001)	0.97 (<0.001)	0.90 (<0.001)	0.41 (<0.001)	0.52 (<0.001)	0.98 (<0.001)	0.67 (<0.001)	1.44 (<0.001)
C			-0.09 (0.228)	0.01 (0.960)	0.17 (0.120)	0.36 (<0.001)	0.68 (<0.001)	0.61 (<0.001)	0.12 (0.201)	0.23 (0.046)	0.69 (<0.001)	0.38 (0.002)	1.15 (<0.001)
D				0.09 (0.423)	0.25 (0.039)	0.44 (<0.001)	0.76 (<0.001)	0.70 (<0.001)	0.21 (0.049)	0.31 (0.002)	0.77 (<0.001)	0.46 (<0.001)	1.24 (<0.001)
AB					0.16 (0.036)	0.35 (0.001)	0.67 (<0.001)	0.61 (<0.001)	0.12 (<0.001)	0.22 (0.034)	0.68 (<0.001)	0.37 (0.012)	1.14 (<0.001)
AC						0.19 (0.082)	0.51 (<0.001)	0.44 (<0.001)	-0.04 (0.618)	0.06 (0.562)	0.52 (0.001)	0.21 (0.171)	0.98 (<0.001)
BC							0.32 (0.030)	0.26 (0.007)	-0.23 (0.011)	-0.13 (0.264)	0.33 (0.014)	0.02 (0.863)	0.79 (<0.001)
BD								-0.07 (0.717)	-0.55 (<0.001)	-0.45 (0.002)	0.01 (0.971)	-0.30 (0.098)	0.47 (0.007)
CD									-0.49 (<0.001)	-0.38 (0.001)	0.07 (0.558)	-0.24 (0.032)	0.53 (<0.001)
ABC										0.10 (0.270)	0.56 (<0.001)	0.25 (0.034)	1.03 (<0.001)
ABD											0.46 (<0.001)	0.15 (0.167)	0.92 (<0.001)
ACD												-0.31 (0.033)	0.46 (<0.001)
BCD													0.77 (<0.001)

† Value in the cell corresponds to *L*(A)-*L*(B)=-0.46–(-0.25)=-0.21, where *L* is the estimated effect; a negative value indicates that the severity of Botrytis bunch rot estimates in the row is lower than that estimates in the column; the probability value of the comparison is in parenthesis.

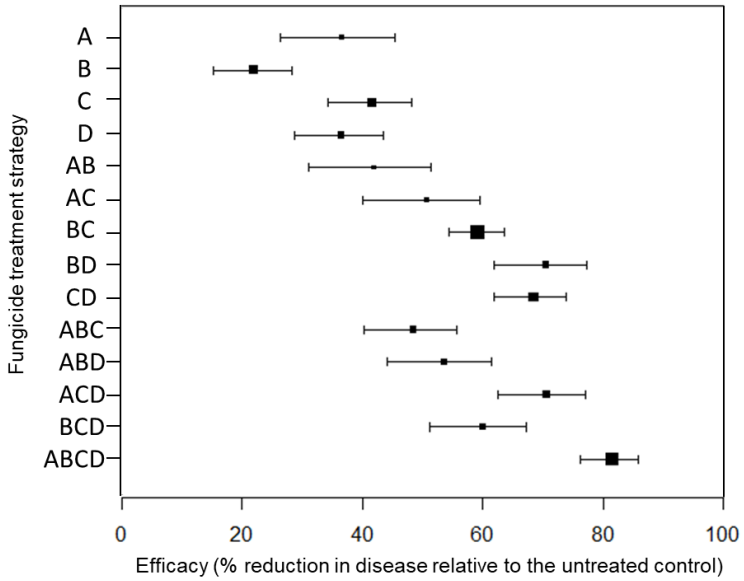

Figure 2.2. Efficacy of different fungicide treatment strategies for the control of Botrytis bunch rot expressed as the percentage of disease reduction relative to the untreated control as estimated by the meta-analysis; whiskers show the 95% confidence interval; the dot size increases with the precision of estimates. Strategies are combinations of treatments applied in A (end of flowering, growth stage 69 of Lorenz et al.1994), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and/or D (before harvest, before growth stage 89).

Table 2.3. Effect in the reduction of Botrytis bunch rot severity of different fungicide classes.

Fungicide class[†]	K[§]	Estimated effect			
		***L*[¥]**	**se(*L*)**	**95% CI (*L*)**	***P***
E3 (intercept)	287	2.86	0.154	2.56 / 3.16	<0.001
C2/G3	6	-0.17	0.802	-1.74 / 1.40	0.829
C5	4	-1.20	0.721	-2.61 / 0.21	0.095
D1	3	-1.09	0.766	-2.59 / 0.41	0.156
D1/C2	6	0.12	0.801	-1.45 / 1.69	0.879
D1/C5	7	-1.35	0.803	-2.92 / 0.22	0.093
D1/C5/G3	7	-0.34	0.800	-1.90 / 1.23	0.675
D1/E2	75	-1.68	0.350	-2.37 / -1.00	<0.001
D1/G3	16	-0.64	0.530	-1.68 / 0.39	0.224
E0	14	0.65	1.115	-1.54 / 2.83	0.561
E3/C5/G3	3	0.37	1.118	-1.82 / 2.56	0.742
E3/D1	5	0.15	0.814	-1.45 / 1.74	0.857
E3/D1/B2	6	0.41	1.115	-1.78 / 2.59	0.714
E3/G1	9	-0.32	0.665	-1.62 / 0.98	0.628
E3/MS	8	-2.33	0.657	-3.61 / -1.04	<0.001
G3	21	-0.73	0.408	-1.53 / 0.07	0.073
MS	3	0.63	1.118	-1.56 / 2.82	0.573
MS/D1/C2	5	0.96	1.116	-1.22 / 3.15	0.387

[†] Fungicides were grouped based on the chemical classes defined by the Fungicide Resistance Action Committee (FRAC 2017).

[§] Total number of studies included in the analysis for each combination of fungicide class.

[¥] Results are presented as the difference (L) in the log mean of disease severity for each fungicide class relative to class E3 (intercept). se= standard error; *CI*= confidence interval; *P* = probability value (significance of the effect in the reduction of the disease).

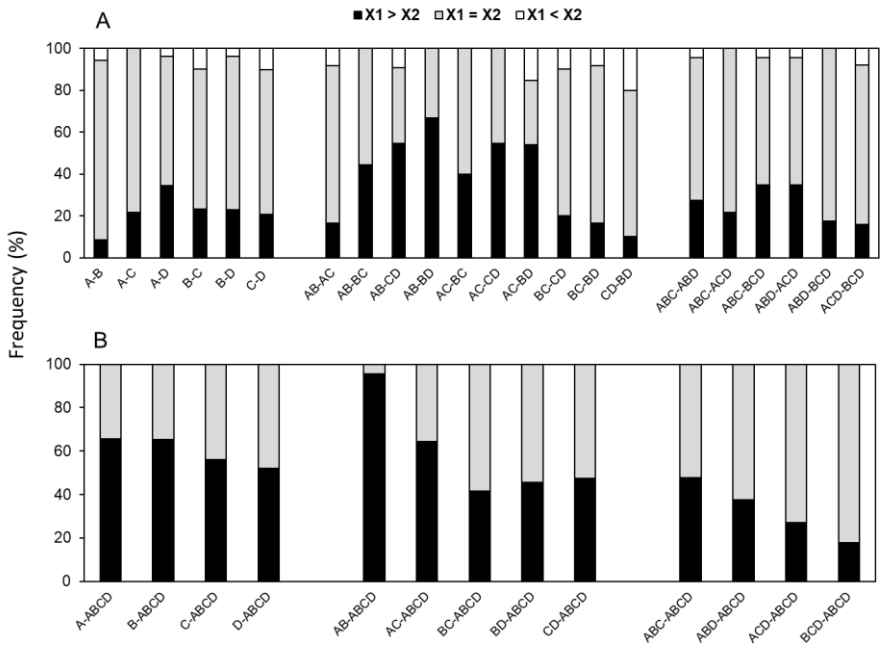

Figure 2.3. Frequency distribution of the studies in which the differences of disease severity between two fungicide treatment strategies for the control of Botrytis bunch rot were significant; white, grey, and black bars indicate the frequency of studies in which severity was less in the first strategy than in the second strategy, equal in both strategies, or greater in the first than in the second strategy, respectively. Strategies are combinations of treatments applied in A (end of flowering, growth stage 69 of Lorenz et al.1994), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and/or D (before harvest, before growth stage 89).

Discussion and conclusion

Since the 1970s, several experiments have been carried out to assess the effectiveness of different fungicides and timings for controlling BBR of grapevines. In these experiments, fungicide strategies were based on the application of sprays during four grape growth stages: A, end of flowering; B, pre-bunch closure; C, veraison; and D, 1 to 3 weeks before harvest (Agulhon 1973; Bisiach et al. 1978a). Most of these experiments were published in national technical journals or were conducted by local institutions with the objective of developing practical recommendations for viticulturists; other experiments remained unpublished. To our knowledge, this paper is the first to summarize the information from those

experiments (from 116 studies) with the aim of drawing robust conclusions (Donegan et al. 2013; Madden and Paul 2011; Scherm et al. 2014).

Meta-analysis was used in the current study, and researchers have expressed the concern that the results of meta-analyses may not be robust because of "publication bias". The concern is that negative results often remain unpublished or are not included in JCR-indexed journals and are therefore less likely to be included in a meta-analysis (Madden and Paul 2011; Scherm et al. 2014). In the current research, publication bias was unlikely for two reasons. First, only 19% of the studies were obtained from JCR-indexed journals; the other 81% were either published in journals/reports not accessible by a systematic review of the main scientific databases or were unpublished. Second, publication source (i.e., JCR-indexed journal, non-JCR journals, or experimental reports) did not affect the results of this work.

There are two additional reasons for considering the findings of this paper robust. First, the database included a wide range of BBR epidemics; disease severity ranged from 0.01% to approximately 90%. Second, the variability in disease severities was caused by among-studies variability rather than by sampling errors within experiments, as indicated by the I^2 statistic (Higgins and Thompson 2002; Madden and Paul 2011); among-studies variability may be mainly related to different environmental conditions that promoted or restricted BBR development (Madden and Paul 2011; Madden and Piepho 2016).

With a 1-spray strategy, BBR control was, on average, more effective when fungicides were applied at timing A, C, or D rather than B. Based on the *B. cinerea* infection pathways defined by Elmer and Michailides (2007), spraying in A (flowering) would simultaneously affect various infection pathways: i) conidial infection of the style and ovules; ii) conidial infection of the stamens or petals; iii) fruit infection via the fruit pedicel; and iv) colonisation of floral debris. Treatments in B (pre-bunch closure), when berries are not susceptible to *B. cinerea* infection (Deytieux-Belleau et al. 2009), have the main aim of disinfesting the colonised floral debris before the debris is enclosed in the growing bunch (Elmer and Michailides 2007). In this meta-analysis, it is therefore not surprising that fungicide sprays were more effective at timing A than B. Later during the season, sprays at timings C and D would reduce the infection of ripening berries caused by both conidial and berry-to-berry infection (Elmer and Michailides 2007; González-Domínguez et al. 2015).

With a 2-spray strategy, strategy AB provided similar disease control as A but better control than B. This result shows that spraying in B after having sprayed in A is not convenient when a 2-spray strategy is used, probably because the two sprays affect the same infection pathway (i.e., the production of inoculum on bunch

trash). Recent results of Calvo-Garrido et al. (2014) (not included in this meta-analysis) confirmed that treatments applied with strategy A vs. AB did not differ in their control of *B. cinerea* on bunch trash and of latent infections at veraison, indicating that a treatment in B did not provide additional control if a treatment had been applied in A. Control was better with strategy AC than AB; unfortunately, AD was not included in this work because only few studies with this strategy were retrieved with the literature search. When the spray in A was missed, strategies BC, BD, and CD provided good control. Therefore, combining treatments affecting both early infection pathways (at timing A or B) and late infection pathways (at timing C or D) results in effective disease control. With a 3-spray strategy, disease control was consistently better with strategy ACD than with BCD and this may be explained based on the effect of A or B on the infection pathways, as described before.

Although the 4-spray strategy, ABCD, provided the best control, it often led to unjustified fungicide applications. The latter inference is supported by Figure 2.3B, which shows that spraying 4 times in ABCD did not always provide better disease control than spraying 1, 2, or 3 times. Therefore, recommending a routine BBR control strategy based on 4 sprays is not justified; it is not profitable for the grower and has negative consequences on human health, environmental pollution, and fungicide resistance management (Alavanja et al. 2004; Epstein 2014; Fillinger and Walker 2016; Leroux 2007).

The inferences and conclusions presented in the previous paragraphs can be considered relevant regardless of the specific fungicides used. Even though fungicide class (defined based on the chemical classes from FRAC; FRAC 2017) had a significant effect on BBR control, only 2 of 18 classes (or combinations of classes) were significantly different from the reference class, the dicarboximides (E3). Control was better with the fungicide combinations D1/E2 (anilino-pyrimidines/phenylpyrroles) and E3/MS (dicarboximides/multi-site) than with E3. Results concerning E3/MS should be interpreted cautiously, because only 3 studies were considered, and all were carried out by the same research group; in those studies, E3/MS may have been more effective than E3 because the *B. cinerea* population may have been resistant to E3, which is a well-known problem (Fillinger and Walker 2016; FRAC 2013; Leroux 2007). Results for D1/E2 (cyprodinil/fluxodinil) may be considered more consistent than those for E3/MS because results for D1/E2 were from 14 studies that were conducted by different research groups. Investigating the efficacy of single fungicides or fungicide combinations against *B. cinerea* was not the aim of the present research.

Given the results of this study and irrespective of the fungicides used, practical recommendations for BBR control should be based on the following

findings: i) strategy A provides better control than B; ii) strategy AC provides similar control as BC (there are no data for a robust comparison of AD vs BD); iii) strategy ACD is slightly better than BCD; and iv) strategy ABCD is useful only when severe epidemics are expected. Therefore, spraying at timing A seems to be very useful for achieving efficient and flexible BBR control in vineyards. Spraying at timing B instead of A does not provide the same flexibility because, if the grower initially decides to adopt a 1-spray strategy and the season subsequently becomes highly favourable for *B. cinerea*, the grower would no longer be able to adopt strategy ABCD. Similarly, the BC or BCD strategies, which are still possible if a spray is not applied at timing A, provide the same control as AC or less control than ACD; strategy BD provides good average control, but comparison with AD was not possible because the latter strategy was not evaluated in this work. If a spray is applied in A, spraying in B is useful only if the grower decides to adopt the ABCD strategy; otherwise, AC or ACD provide satisfactory solutions for 2- or 3-spray strategies, respectively.

In some viticultural areas, spraying at timing A has been considered much less effective than spraying in B. After conducting a 2-year experiment in which strategy ABCD provided the same control as BCD, Corvi and Tullio (1980) proposed to eliminate the spray in A; however, in both years of that experiment, the environmental conditions during flowering were unfavourable for BBR development. Pérez-Marín (1998) recommended strategy BC based on a 4-year experiment in the same vineyard, but no statistical analysis was provided. In Italy and Spain, most of the regional public sanitary services recommend spraying in B rather than in A to their viticulturists (CODILE 2017; Costacurta et al. 2004; DOG 2005; IRVV 2017). On the other hand, some recent papers emphasize the importance of spraying at timing A rather than B (Calvo-Garrido et al. 2014; Ciliberti et al. 2015a, González-Domínguez et al. 2015).

Results of this work provide information on the efficacy of different BBR control strategies based on 1, 2, 3, or 4 sprays per season. How many sprays are necessary to control BBR in a vineyard may depend on several factors, including weather conditions, the susceptibility level of the variety, the microclimate as influenced by the canopy structure and density, and presence of powdery mildew and berry moth insects (Elad et al. 2007). This decision can clearly be made easier by use of a mathematical model that is able to predict the risk of the disease development. A recently published mechanistic model for *B. cinerea* (González-Domínguez et al. 2015) predicts, on a daily basis, the relative infection severity during two infection windows corresponding to the two grape-growing periods relevant for *B. cinerea* infection: i) between “inflorescences clearly visible” and “berries groat-sized, bunches begin to hang”; and ii) ripening berries (Elmer and

Michailides 2007). The model, which is based on relative infection severity values, predicts the final BBR as light, intermediate, or severe. The model has been integrated in a Decision Support System (DSS) for the sustainable management of vineyards and is therefore available for growers (Caffi et al. 2017). The findings of the current study, combined with the model predictions, should improve BBR management in vineyards.

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Supplementary material

Meta-Analysis procedure

For each study, disease severity data (*X*) from fungicide-treated (T) and untreated plots (NT) were used to estimate the treatment effects. The log mean severity of treatments, i.e.,$\ln(\bar{X})$ was calculated as the effect size for each study and strategy. The log severity of treatments was used because ln(*X*) instead of *X* because its distribution is closer to the normal one, which is required by the model used for the meta-analysis (see below).

The within-study variance was estimated for each treatment of a single study as $s^2=V/(n\bar{X}^2)$, where *V* is the residual error component of the study, *n* is the number of replicates, and $\bar{X}$ is the mean disease severity. To calculate *V* for each study, different approaches were used (Ngugi et al. 2011a; Paul et al. 2007): i) when the original data were available, *V* was extracted directly from the ANOVA table of the study (i.e., the residual variance or mean square error); ii) when the study did not include the ANOVA table but did include the least significant difference (LSD), *V* was calculated as (n (LSD/1.96)^2/2); iii) when only the significant mean separation was provided (i.e., significant differences between means were denoted by letters in a graph or a table), the estimated LSD was computed as the average between the smallest observed significant difference and the largest observed non-significant difference; iv) when *V* was not available and the mean separation had not been calculated by LSD (this occurred in 54 studies), *V* was estimated based on the relationship between *V* and mean $\bar{\bar{X}}$ observed in the other 62 studies, where ($\bar{\bar{X}}$) is the average of mean across all the treatments of each study (including the untreated control). In the latter case, a power-law model was fitted to the disease severity data as $\ln(V) = a + b \cdot \ln(\bar{\bar{X}})$ to calculate *V* for the studies with missing variance information; the power-law model provided highly significant fits to sampling variance with a=0.226±0.227, b=1.385±0.088, R^2=0.834, and P<0.001.

The meta-analysis was conducted using the software R (v 3.4.0; package 'metafor'; CoreTeam R 2017; Viechtbauer 2010). A multivariate random effects model was fitted, via linear (mixed-effects) models, by using the rma.mv function of the 'metafor' package4. The model was fitted in the form $Y \sim N(\mu, \Sigma+S)$, where $\sim N$ indicates a multivariate normal distribution, μ is the expected value for the different treatments, Σ is the between-study variance-covariance matrix, and *S* is the within-study variance-covariance matrix.

To run the model, the $\ln(\bar{X})$ and the corresponding sampling variances (within-study sampling variance; s^2) were specified in the arguments of the function rma.mv. The restricted maximum-likelihood estimation (REML) method was used

for model fitting. An unstructured matrix was selected for Σ (Machado et al. 2017; Paul et al. 2008); for *S*, the diagonal elements were the s values for each treatment and study, and the off-diagonal elements were 0 (Paul et al. 2008). Random effects were specified in the form ~ inner|outer, with the outer factor corresponding to the study identification and the inner factor corresponding to the treatment type (i.e., the strategy; Machado et al. 2017).

To evaluate model assumptions, heterogeneity and consistency were considered (Donegan et al. 2013). A test for residual heterogeneity (Q_E) was conducted with the rma.mv function, that evaluate whether the variability in the observed effect that is not accounted by the moderators included in the model is larger than one would expect based on sampling variability alone. To assess the nature of the residual heterogeneity, I^2 statistics were calculated as proposed by Higgins and Thompson (2002) and Jackson et al. (2012). Prior to perform the analysis, a similar model of that described, but with fixed effects was calculated. For each treatment, I^2 statistic was based on the relation between the variance-covariance matrix of models with fixed and random effects, in the form: (vcov(random)[1,1]-vcov(fixed)[1,1]) / vcov(random)[1,1]). Different positions of the diagonal of the matrix were selected for the different treatments. To assess consistency, an independent analysis was run as proposed by Madden et al. (2016) and Piepho et al. (2015). Two consistency groups were created, one with the cases directly obtained from the studies and the other with the cases inferred. A significant interaction of the treatment and consistency groups, evaluated based on the Wald test statistic, was an indication of inconsistency.

Results were presented as the difference, *L*, in the effect sizes for each T treatment relative to the untreated control NT, in the form $L_T=ln(X_T)-ln(X_{NT})$. *L* was used instead of the response ratio (ratio of the means in the treatment and control) to avoid functional correlations between effect sizes within a given study arising from a common denominator (Ngugi et al. 2011a). Standard errors and confidence intervals were likewise calculated for these values of *L*. A Wald-type test statistic was used to determine whether the mean differences *L* were significantly different from zero, i.e., whether the disease severity in the treated plots $ln(X_T)$ differed from that in the untreated plots $ln(X_{NT})$. The percentage of disease reduction relative to the control was also estimated as (1− exp(*L*)) x 100, and the 95% confidence intervals were calculated as in Ngugi et al. (2011b) and Paul et al. (2008).

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Chapter 3

Chapter 3

Interactions among fungicides applied at different timings for the control of Botrytis bunch rot in grapevine[1]

Abstract

Botrytis bunch rot (BBR), caused by *Botrytis cinerea*, is one of the main diseases affecting grapevines. Due to the complexity of the *B. cinerea* life cycle and the existence of different infection pathways affecting grapevine tissues at both early and late growth stages, fungicides are usually applied sequentially at the end of flowering (A), pre-bunch closure (B), veraison (C), and before harvest (D). Interactions among fungicides (from different groups) applied at these growth stages were evaluated in this work, with focus on the strategies in which early- and late-season applications are combined (i.e., strategies AB, CD, ABC, ABD, ACD, BCD and ABCD). The evaluation was performed in a set of 116 studies carried out in different years and locations, by comparing the observed (b_{obs}) and expected (b_{pred}) efficacies in controlling BBR; b_{obs} was calculated as the reduction of BBR severity in treated plots compared to untreated ones, while b_{pred} was calculated by using a mathematical function. Early-season sprays (i.e., A and B) showed non-additive interactions (i.e., the observed efficacy was significantly lower than expected in case of additive effect) while late-season sprays (i.e., C and D) did. No significant synergistic effects were observed among fungicide sprays. In the early-season, spraying in A was more effective than in B, and both sprays (A and B) were useful under high disease pressure only, when the full ABCD strategy was needed for effective BBR control. Otherwise, the most effective combination was ACD, able to exploit the additive control of the early-season infection pathways and the multiple infection events during berry ripening.

[1] González-Domínguez E, Fedele G, Languasco L, Rossi V, 2019. Crop Protection 120:30-33.

Introduction

Botrytis bunch rot (BBR), one of the main diseases affecting grapevines, is caused by *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel). The fungus affects more than 200 plant species; on grapevine, it may cause significant losses, and markedly reduces the quality of the wines (Elad et al. 2007; Ribereau-Gayon et al. 1980).

Multiple infection pathways of *B. cinerea* have been described, occurring in two periods (Elmer and Michailides 2007): in the early season, from inflorescence clearly visible and when berries are groat sized (corresponding to growth stages 53 and 73 of Lorenz et al. 1995, respectively), and in the late season, from veraison to berry maturity (growth stages 79 and 89, respectively). In the early season, conidia cause infection of inflorescences and young berries through diverse pathways (infection of the style and ovule, stamens or petals, or young fruit infection via the pedicel; Elmer and Michailides 2007). These infections cause either inflorescence blight or they develop into latent infections of berries, which result in berry rot after veraison (Holz et al. 1997; Keller et al. 2003; McClellan and Hewitt 1973). During the flowering stage, the pathogen also colonizes saprophytically bunch trash (aborted flowers, calyptras and stamens), which are retained within the developing bunches; this saprophytic mycelium has been considered a major source of inoculum for late season infections (Nair and Parker 1985). After veraison, a classical pre-harvest polycyclic epidemic can develop under favourable weather conditions; rot develops, and new infections can be caused by both conidia or mycelium form the adjacent infected berries (berry-to-berry infections) (Elmer and Michailides 2007; González-Domínguez et al. 2015).

Due to the complexity of the life cycle of *B. cinerea*, growers usually apply fungicides at four specific grape growth stages: A, end of flowering (growth stage 69); B, pre-bunch closure (growth stage 77); C, veraison (growth stage 83); and D, before harvest (before growth stage 89) (Baldacci et al. 1962; Bulit et al. 1970). This calendar schedule is easy to follow and provides good protection against BBR (Agulhon 1971; Lafon et al. 1972). However, it may result in unnecessary sprays with damaging consequences on environment, public health and the rise of fungicide resistance (Epstein 2014; Panebianco et al. 2015). Several works have then studied the key timings for fungicide applications with the objective of reducing the number of sprays; a number of these works have been recently reviewed quantitatively in a network meta-analysis by González-Domínguez et al. (2019, see Chapter 2). Results of this meta-analysis showed that, when only one spray is applied, BBR control is more effective when fungicides are applied in A, C, or D than in B. For strategies with more than one spray, CD and ACD provide

good disease control; the full control strategy ABCD is profitable only in case of severe epidemics.

An interesting research question is whether and, if so, how repeated fungicide applications interact one to each other. This area has been investigated for fungicide mixtures, and it is known that the biological effect (i.e., the control of the target pathogen) of a fungicide mixture may be equal, greater or smaller than the effect of the single fungicides administered separately; these interactions have been defined as additive, synergistic and antagonistic (or non-additive), respectively (Gisi 1996; Levy et al. 1986). Whether the fungicides affect the development of the target organism simultaneously or separately over time has also been considered, which depends on the specific physiological activity, or vital system, or growth stage of the pathogen affected by each fungicide; for example, one fungicide affects spore germination, and another affects mycelial growth but not spore germination. Based on this approach, the following cases have been defined: the fungicides have different simultaneous action, similar simultaneous action or nonsimultaneous action (Kosman and Cohen 1996). Mathematical formulas have been developed to study these interactions, and they were used for different target organisms (Abbott 1925; Colby 1967; Drury 1980; Gisi 1996; Kosman and Cohen 1996; Wadley 1945).

Paveley et al. (2003) adapted these formulas to study the efficacy of fungicide spray programmes in which two treatments are applied sequentially. In this function, a second application is considered to act on that proportion of the total pathogen population that was uncontrollable at the time of the first application plus any additional part of the population that survived the first application as a result of a finite dose being applied. Paveley et al. (2003) also speculated that this equation for two sprays might be expanded to predict the combined efficacy of multiples sprays.

In this work, an expanded version of the function of Paveley et al. (2003) was used to evaluate the interactions among fungicide treatments applied at different timings for the control of BBR in vineyards. The work focuses on those strategies in which treatments are combined in such a way to control different infection pathways, applied in early (A and/or B) and late (C and/or D) season. Specifically, the combinations of early-season (AB) vs. late-season (CD), and strategies in which early- and late-season applications are combined (ABC, ABD, ACD, BCD and ABCD) were considered. To assess the interaction among these fungicide strategies, observed and expected efficacies in controlling BBR were compared, and their ratio was evaluated.

Materials and methods

Observed efficacy of the different treatment strategies

The observed efficacy (b_{obs}) of the fungicide strategies evaluated in this work (A, B, C, D, AB, CD, ABC, ABD, ACD, BCD and ABCD) was calculated by a meta-analysis carried out by González-Domínguez et al. (2019, see Chapter 2) by using 116 different studies. Details on fungicide applications and disease assessments were provided in González-Domínguez et al. (2019, see Chapter 2). In short, the database for the meta-analysis included experiments in which BBR severity was assessed in studies with at least one fungicide (applied at timings A, B, C, or D, or at their combinations), and an untreated control (NT). The database was built with a wide range of epidemics with BBR severity in the NT plot from 0.1% to 87.4%. The active ingredient/s used varied among studies, and were grouped based on the chemical class as defined by the Fungicide Resistance Action Commite, FRAC (González-Domínguez et al. 2019, see Chapter 2; FRAC, 2018). Dicarboxamides were the most used fungicides in the above studies (60% of the studies) and are the most used ones worldwide (Williamson et al. 2007); therefore, they were considered as the reference group in the meta-analysis to test the effect of different fungicide groups on BBR control.

The values of b_{obs} for each fungicide strategy were calculated as $b_{obs} = 1 - \beta$; β is the slope of the linear regression between the BBR severity at harvest in treated (D) and the untreated control (D_0) in the form $D = \beta \times D_0$. Both severities, D and D_0, are in a 0-1 scale where 1 = 100% disease severity. Values of b_{obs} and their 95% confidence intervals are shown in Table 3.1; these values range between 0.22 (for B) and 0.81 (for ABCD), and the correlation coefficients range between 0.67 (for ABCD) and 0.98 (for B). Figure 3.1 shows, for the example of A, AB, ABC, and ABCD, the regression lines and how the b_{obs} increases with the number of fungicide sprays.

Table 3.1. Observed and predicted efficacy of fungicide strategies for controlling Botrytis bunch rot in vineyards, and summary statistics for assessing their interactions.

Fungicide treatment strategy[a]	Observed efficacy (95% CI)[b]	*r*[c]	Predicted efficacy (95% CI)[d]	*P*[e]	Ratio b_{obs}/b_{pred} [f]
A	0.37 (0.26-0.45)	0.96			
B	0.22 (0.15-0.28)	0.98			
C	0.42 (0.48-0.34)	0.94			
D	0.36 (0.29-0.43)	0.93			
AB	0.42 (0.31-0.51)	0.94	0.51 (0.38-0.61)	0.08	0.83
CD	0.68 (0.62-0.74)	0.89	0.63 (0.53-0.71)	0.91	1.08
ABC	0.48 (0.40-0.55)	0.91	0.72 (0.59-0.81)	0.03	0.67
ABD	0.53 (0.44-0.61)	0.83	0.69 (0.56-0.79)	0.16	0.77
ACD	0.71 (0.62-0.77)	0.90	0.78 (0.66-0.85)	0.07	0.91
BCD	0.60 (0.51-0.67)	0.88	0.72 (0.61-0.80)	<0.001	0.83
ABCD	0.81 (0.76-0.86)	0.67	0.83 (0.72-0.90)	0.62	0.98

[a] Fungicide strategies are based on 1, 2, 3, or 4 fungicide sprays applied at timings A (end of flowering, growth stage 69 of Lorenz et al.1995), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and/or D (before harvest, before growth stage 89).
[b] 1 minus the regression coefficients (and their 95% confidence intervals) between the BBR severity at harvest in treated and the untreated control. Data from the meta-analysis developed by González-Domínguez et al. (2019, see Chapter 2).
[c] Pearson correlation coefficients for the linear regression between the Botrytis bunch rot severity in the untreated control (independent variable) and in the fungicide treatment strategies (dependent variable).
[d] Efficacy predicted in this work using equation (2).
[e] Probability value of the *t* test conducted to assess the differences between observed and predicted efficacy.
[f] Ratio between observed and predicted efficacy.

Expected efficacy of the different treatment strategies

An expanded version of the function of Paveley et al. (2003) was used to predict the efficacy of repeated applications of fungicides in controlling BBR severity, in the following form:

$$D = D_0 \times \prod_{t=1}^{n}\left[1 - b_t \times \left(1 - e^{-k \times d_t}\right)\right] \quad (1)$$

where: D and D_0 are BBR severity in the treated and the untreated control (dose = 0), respectively; $\prod$ indicates a product, i.e., the multiplication of the terms in brackets for the number t of treatments applied (with t ranging from 1 to n, with n=2, 3 or 4); b is the amount of disease that might potentially be controlled by the treatment t with an infinite dose (i.e., the efficacy of the treatment expressed as a

proportion of D_0); k is the rate of change of disease severity with dose, which is expressed as dose^{-1}, and increases as the dose decrease; and d_t is the dose used for each treatment. For example, for strategy ACD, the equation is as follows:

$$D = D_0 \times [1 - b_A \times (1 - e^{-k \times d_A})] \times [1 - b_C \times (1 - e^{-k \times d_C})][1 - b_D \times (1 - e^{-k \times d_D})] \quad (2)$$

To calculate the value of D for each strategy (i.e., AB, CD, ABC, ABD, ACD, BCD, and ABCD) values of b for the different 1-spray applications, b_A, b_B, b_C, and b_D, were the b_{obs} for A, B, C and D, respectively, calculated as previously described (Fig. 3.1); d was set d=1, because all the fungicides were applied at the label dose. The parameter k was estimated by using the data from Markoglou and Ziogas (2002), referring to the efficacy of different SBIs (sterol biosynthesis inhibitors) fungicides against *B. cinerea* on cucumber seedlings, namely fenpropimorph, pyrifenox, flusilazole, triflumizole, and propiconazol. In this research, the efficacy was assessed as the reduction of disease severity compared to an untreated control, after artificial inoculation of *B. cinerea* conidial suspensions and incubation under optimal conditions; fungicides were applied at different dosages from 25% to 200% of the label dose. In this work, it was assumed that the k values calculated for cucumber seedlings and grape berries do not change, as it has been shown that botryticides have similar mode of action, efficacy and resistance mechanisms in different host plants of *B. cinerea* (Fillinger and Walker 2016; Leroux 2007; Rosslenbroich and Stuebler 2000).

The values of k for each fungicide and for the overall fungicides were estimated by fitting the experimental data of disease severity D to equation (1) (with t=1), using the function nls of the 'stats' package of R v. 3.4.0 (R Core Team 2017). Results are shown in Table 3.2. For the overall fungicides, k was estimated as k = 3.37±0.25. Since SBI fungicides did not show any significant effect in comparison to dicarboxamides and most of the fungicide groups used against *B. cinerea* (González-Domínguez et al. 2019, see Chapter 2), this value of k can be considered valid for a wide number of fungicides used against BBR.

The expected efficacy (b_{pred}) for each fungicide strategy was calculated from equation (1) in the form:

$$b_{pred} = \frac{1-(\frac{D}{D_0})}{1-e^{-k \times d}} \quad (3)$$

where: d=1 and k = 3.37. Values of b_{pred} (with their 95% Cis) are shown in Table 3.1.

Table 3.2. Estimation of the parameter *k* for different fungicides used for controlling *Botrytis cinerea*.

Fungicides	k^a	SE	R^2
Fenpropimorph	6.72	0.49	0.996
Flusilazole	3.11	0.19	0.994
Propiconazole	2.49	0.10	0.991
Pyrifenox	4.01	0.39	0.987
Triflumizole	2.21	0.16	0.992
Overall	3.37	0.25	0.950

[a] *k* was estimated by fitting the experimental data of disease severity *D* from Markoglou and Ziogas (2002) to equation (1) (with *t*=1). SE= standard error; R^2= coefficient of determination.

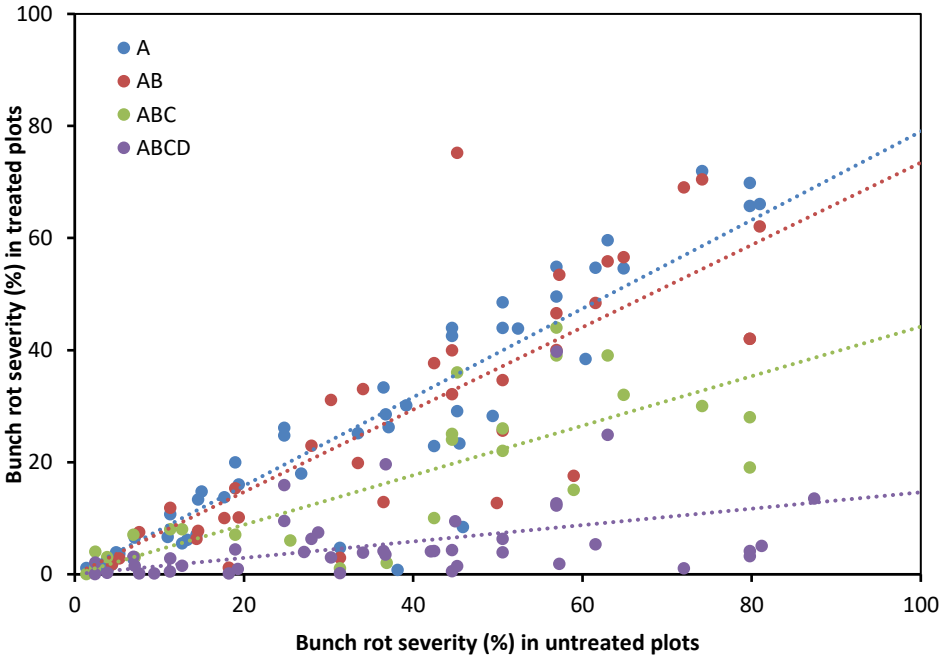

Figure 3.1. Linear regression (y=(1-b_{obs})x) between Botrytis bunch rot severity in the untreated control (x) and in four fungicide treatment strategies (y) (see Table 3.1 for the regression parameters); dots are the observed disease severities in the meta-analysis of González-Domínguez et al. (2019, see Chapter 2). Strategies are combinations of treatments applied in A (end of flowering, growth stage 69 of Lorenz et al. 1995), B (pre-bunch closure, growth stage 77), C (veraison, growth stage 83), and D (before harvest, before growth stage 89).

Interaction among fungicide applications

To assess the interaction among fungicides applications, the ratio between observed and expected efficacy, b_{obs}/b_{pred}, was calculated for each strategy; when b_{obs}/b_{pred} =1 the effect of each treatment is considered as additive, when > 1 the effect is more than additive (synergistic) and when < 1 is less than additive (non-additive) (Gisi 1996; Levy et al. 1986). Whether the difference between b_{obs} and b_{pred} was statistically significant was tested with a *t* test. The *t* test was conducted for two normal populations generated randomly from the observed and predicted efficacy of each strategy (using the functions rnorm and t.test of the 'stats' package of R v. 3.4.0); standard deviations (SD) were calculated from the 95% CI. Values of b_{obs}/b_{pred} were considered =1 (additive) when b_{obs} and b_{pred} were not statically different one from each other at $P \geq 0.01$, 0.05 or 0.1; otherwise, there were considered significantly < or > 1 (i.e., non-additive and synergistic, respectively).

Results and discussion

Interaction were non-additive for some fungicide strategies, and additive for others (Table 3.1). No synergistic effects were observed for any of the strategies evaluated; i.e., the efficacy observed in the field was never significantly higher than that predicted. Significant non-additive effects were observed for AB (b_{obs}/b_{pred} = 0.83, P=0.08), ABC (b_{obs}/b_{pred} = 0.67, P=0.03) and BCD (b_{obs}/b_{pred} = 0.83, P<0.001). Concerning AB, Gonzalez-Dominguez et al. (2019, see Chapter 2) discussed that both treatments, in A and B, affect the same infection pathway, i.e., the colonisation of bunch trash (Elmer and Michailides 2007). Treatment in A also affects the latent infection pathway, and this may justify the higher efficacy of A (b_{obs} = 0.34) compared to B (b_{obs} = 0.22). This also agree with previous results in which the strategy AB provide similar control than A (Calvo-Garrido et al. 2014; González-Dominguez et al. 2019, see Chapter 2).

The non-additive effect of the strategy ABC may be related to the non-additive effect of AB. The ratio b_{obs}/b_{pred} = 0.77 of the strategy ABD confirms that AB does not provide additive effects. For ABD the difference between b_{obs} and b_{pred} was not significant (P=0.16) due to high variability in the estimation of both b_{obs} and b_{pred} (Table 3.1). The non-additive effect for BCD show that the treatment in pre-bunch closure (B) does not increase the efficacy of the late-season treatments CD, being b_{obs} = 0.6 for BCD and = 0.68 for CD. A slight non-additive effect was also found for ACD, with b_{obs}/b_{pred} = 0.91 (P=0.07), but the observed efficacy was, however, rather high, with b_{obs} = 0.71.

Additive effects were observed for CD (b_{obs}/b_{pred} = 1.08, P=0.91) and ABCD (b_{obs}/b_{pred} = 0.98, P=0.62). ABCD was the most effective strategy, with b_{obs} = 0.816. Concerning CD, both treatments affect the same pathway (infection of ripening

berries) and therefore no-additive effects might be expected. The observed additive effects may be related to the long time elapsing between veraison (C) and pre-harvest (D), and to the classical polycyclic pattern of BBR epidemics in this period (Elmer and Michailides 2007). The conidia of *B. cinerea* are generally present in the vineyard during grape ripening (Rodríguez-Rajo et al. 2010; Vercesi and Bisiach 1982) and environmental conditions are frequently conducive being the pathogen able to cause infection under a wide range of temperature and moisture conditions (Ciliberti et al. 2015; Nair et al. 1988). Moreover, the susceptibility to *B. cinerea* of berries considerably increases with maturity (Deytieux-Belleau et al. 2009). Thus, spraying in C and D may enable the control of repeated infections during the grape ripening period.

In conclusion, the present work increases our knowledge about the interaction of repeated fungicide applications against BBR in vineyards. Results of this work indicated that the decision about fungicide application would benefit from an analysis of the infection pathways the different sprays are able to affect, considering that the subsequent application of fungicides controlling the same pathway is not fully beneficial in the early-season, as for AB. Spraying in A is more convenient than in B, and performing both sprays is only recommended under conditions of high BBR pressure, in which the strategy ABCD should be recommended. Otherwise, strategies CD or ACD are the most convenient, being able to exploit the additive effect of the interventions on repeated infection events (for CD) or different infection pathways of *B. cinerea* (for ACD).

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Chapter 4

Chapter 4

Quantification of *Botrytis cinerea* in grapevine bunch trash by Real-time PCR[1]

Abstract

Quantification of colonization of grape bunch trash by *Botrytis cinerea* is crucial for Botrytis bunch rot (BBR) control. A previously developed qPCR method was adapted to quantify *B. cinerea* DNA in grape bunch trash, and a colonization coefficient (CC) was calculated as the ratio between the DNA concentrations of *B. cinerea* and of *Vitis vinifera.* CC values increased linearly with the number of conidia of *B. cinerea* or the quantity of mycelium of *B. cinerea* added to the bunch trash increased. CC values also increased linearly in bunch trash samples containing increasing percentages of *B. cinerea*-colonized bunch trash; in the latter samples, CC values were correlated with subsequent assessments of *B. cinerea* colonization of trash (as determined by plating on agar) and sporulation on the trash (as determined by spore counts after incubation in humid chambers). The qPCR assay was also validated using trash collected from bunches treated or not treated with fungicides in three vineyards in 2 seasons. CC values reflected the reduction in sporulation and in latent infections of mature berries caused by fungicide application. The qPCR assay enables rapid, specific, sensitive, and reliable quantification of the degree of colonization of bunch trash by *B. cinerea*, which makes it a useful tool for studies of the epidemiology and management of BBR.

[1] Si Ammour M, Fedele G, Morcia C, Terzi V, Rossi V, 2019. Phytopathology 109:1312-1319.

Introduction

Botrytis bunch rot (BBR) is an economically important disease of grapevines (*Vitis vinifera* L.) and is caused by the fungus *Botrytis cinerea* Pers.: Fr (Elmer and Michailides 2007). Control of BBR is challenging for the following reasons: i) *B. cinerea* can function as a saprophyte, necrotroph, or parasite; ii) the fungus can overwinter and sporulate on multiple inoculum sources (including bunch trash, leaf trash, and rotted berries); iii) grapevines are susceptible at multiple growth stages; iv) multiple infection pathways exist; and v) infections can occur under a wide range of environmental conditions, which differ among infection pathways (Ciliberti et al. 2015a; 2015b; 2016; Elad et al. 2007; Elmer and Michailides 2007; Hill et al. 2014; Nair et al. 1995).

After flowering, the pathogen is able to saprophytically colonize the "bunch trash", i.e., the dead stamens, aborted flowers, aborted berries, calyptras, tendrils, and leaf pieces retained within developing bunches (Seyb et al. 2000). Under favorable conditions, the fungus produces abundant conidia on the colonized bunch trash, and these conidia are a source of inoculum for berry infection, mainly after veraison. Elmer and Michailides (2007) referred to these phenomena as infection pathway III (conidial infection and extensive colonization of floral debris in grape bunches), IV (conidial accumulation within the developing bunch), and V (conidial infection of ripening fruit), respectively.

Bunch trash colonized early by *B. cinerea* is a major source of berry infection (Elmer and Michailides 2007), and has been related to the severity of BBR at harvest (Holz et al. 2003; Keller et al. 2003; Nair et al. 1995; Viret et al. 2004). Thus reducing the quantity of bunch trash, and reducing colonization of bunch trash by *B. cinerea* at flowering and post-flowering, should contribute to control of BBR (Calvo-Garrido et al. 2014; González-Domínguez et al. 2015). Reduction of available bunch trash has been explored by removal of floral debris from clusters either at early or at late fruit set using compressed air or leaf blowers (Wolf et al. 1997), and fungicide sprays (González-Domínguez et al. 2015) and the application of biocontrol agents and other natural products (Calvo-Garrido et al. 2014; Pertot et al. 2017) have been investigated to reduce colonization of bunch trash by *cinerea*.

Evaluation of the effectiveness of these interventions requires methods to quantify the colonization of bunch trash by *B. cinerea* and the subsequent production of spores. Traditionally, *B. cinerea* colonization of bunch trash has been quantified by plating on selective media (Abdelwahab and Younis 2012; Edwards and Seddon 2001) or by microscopic assessment (Calvo-Garrido et al. 2014). Sporulation on bunch trash was measured using a sporulation index on a 0–5 scale (Calvo-Garrido et al. 2014) or by microscope counts of conidia (Jaspers et al. 2013;

Keller et al. 2003; Mundy et al. 2012; Nair et al. 1995). As alternatives to traditional methods, molecular tools may offer rapid, specific and accurate estimation of the quantity of *B. cinerea* in bunch trash (Diguta et al. 2010; Abdelwahab and Younis 2012).

A direct polymerase chain reaction (PCR) assay has been described for the detection of *B. cinerea* in pea-sized berries and receptacles (Gindro et al. 2005). Real-time quantitative PCR (qPCR) assays have been developed for the detection and quantification of *B. cinerea* inoculum (conidia and/or mycelium) from air samples and the surface of ripe berries (Carisse et al. 2014; Diguta et al. 2010), and for the quantification of the colonization of *B. cinerea* in developing grape berries and receptacles (Cadle-Davidson 2008; Saito et al. 2013), grape stamens and ripe berries (Celik et al. 2009; Hill et al. 2014; Sanzani et al. 2012).

The objectives of the current study were to: (i) optimize a hydrolysis probe-based qPCR assay for the quantification of *B. cinerea* DNA in bunch trash; (ii) investigate the relationships between the quantity of *B. cinerea* DNA measured by qPCR, and the colonization measured based on the plating method, and as measured by sporulation potential of bunch trash using microscope counts of conidia; and (iii) evaluate the qPCR assay under vineyard conditions.

Materials and methods

Real-time qPCR optimization

Fungal isolates. Strains of *B. cinerea* belonging to the transposon genotypes *transposa* (isolate 213 T) and *vacuma* (isolate 351 V) (Ciliberti et al. 2016), and other fungal isolates (Table 4.1) were obtained from the culture collection of the Department of Sustainable Crop Production of the Università Cattolica del Sacro Cuore (UCSC), Piacenza, Italy. The biotrophic pathogens *Plasmopara viticola* and *Erysiphe necator* were collected from symptomatic leaves (cv. Merlot) in the vineyard of UCSC in 2017 and 2018, and were maintained on container-grown grape plants (cv. Merlot) in a greenhouse kept at a temperature of 24±3°C and 12 h photoperiod.

Plant material. Bunch trash was obtained from plants grown in a greenhouse to minimize natural colonization by *B. cinerea*. Woody cuttings were collected in winter from an experimental vineyard (*V. vinifera* cv. Merlot) at the Università Cattolica del Sacro Cuore. Cuttings were grown in a greenhouse maintained at 24±3°C and 12 h photoperiod, and flowers were obtained following the technique of Mullins and Rajaskekaren (1981). At full flowering, bunch trash was collected by gently shaking the inflorescences inside paper bags. Bunch trash samples were desiccated at 35-40°C for 72 h, and the dry weights determined. Samples were stored at room temperature until use.

DNA extraction. Genomic DNA was obtained from 15 fungal species (Table 4.1) and bunch trash samples. Except in the case of *P. viticola* and *E. necator*, genomic DNA was extracted from fresh mycelium (obtained by scraping the surface of 10 day-old-colonies grown on potato dextrose agar [PDA], at 20°C and 18h photoperiod). DNA of *P. viticola* and *E. necator* was obtained from leaf discs with lesions showing abundant and fresh sporulation (100 mg of leaf material). In brief, mycelium (100 mg fresh weight) or bunch trash (100 mg dry weight) was placed in 2 ml microcentrifuge tubes containing 100 mg of glass sand (425-600 µm diameter), two glass beads (5 mm diameter), and 500 µl of cetyl trimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM ethylenediaminetetraacetic acid [EDTA], 1.4 M NaCl, and 1% polyvinylpyrrolidone [PVP]). The tubes were placed in a Mixer Mill MM200 (Retsch GmbH, Haan, Germany) for 1 min at 30 cycles/s. Subsequently, a CTAB DNA extraction procedure was conducted as described by Saito et al. (2013). The yield and purity of the extracted DNA were determined using a NanoDrop™2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The extracts were adjusted to 10 ng/µl of DNA for fungal samples and to 20 ng/µl of DNA for bunch trash samples.

Primers and hydrolysis probes. The qPCR assay was based on two specific primers and a hydrolysis probe (Bc3) designed to target the intergenic spacer region (IGS) of the nuclear ribosomal DNA (Suarez et al. 2005) of *B. cinerea*. To normalize the quantification DNA of *B. cinerea* in plant tissues, two specific primers and a hydrolysis probe (Res) designed to target the *V. vinifera* resveratrol synthase gene I (Valsesia et al. 2005) were used as an internal control, with the fluorescent reporter JOE as a substitute for FAM (6-carboxyfluorescein). The sequences for Bc3 were as follows: 5′-GCT GTA ATT TCA ATG TGC AGA ATC C-3′ (forward [Bc3F]); 5′-GGA GCA ACA ATT AAT CGC ATT TC-3′ (reverse [Bc3R]); 5′-6-FAM-TCA CCT TGC AAT GAG TGG-BHQ-1-3′ (probe [Bc3P]). The sequences for Res were as follow: 5’-CGA GGA ATT TAG AAA CGC TCA AC-3’ (forward [ResF]); 5’-GCT GTG CCA ATG GCT AGG A-3’ (reverse [ResR]); and 5’-JOE-TGC CAA GGG TCC GGC CAC C-TAMRA-3’(probe [ResP]).

Singleplex and duplex reactions. Singleplex reaction mixtures contained 1x QuantiTect Multiplex PCR Kit (Qiagen, Milano, Italy), 150 nM of probe (Bc3P or ResP), 500 nM of each primer (Bc3F/R or ResF/R), and 2 µl of DNA template in a final volume of 10 µl. Duplex reaction mixtures contained 1x QuantiTect Multiplex PCR Kit, 150 nM of the *V. vinifera* probe ResP, 150 nM of the *B. cinerea* probe Bc3P, 100 nM of each *V. vinifera* primer (Res F/R), 500 nM of each *B. cinerea* primer (Bc3F/R), and 2 µl of DNA template in a final volume of 10 µl. Both singleplex and duplex assays were performed using an Applied Biosystems 7300

Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA) with an initial incubation at 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s.

Specificity. The specificity of the qPCR assay for the detection of *B. cinerea* in grape tissue was determined in a test that included the most common grape pathogens and other fungal species frequently found in grapevines and in air samples (Table 4.1); several of these species were not assessed by Suarez et al. (2005).

Standard curves, calibration DNA, and qPCR optimization. Standard curves were obtained from two singleplex qPCR assays: the Res assay with DNA of *V. vinifera* from bunch trash as template in a 10-fold dilution series (from 20 to 0.02 ng/µl), and the Bc3 assay with DNA of *B. cinerea* extracted from the mycelium (isolate 213T, Ciliberti et al. 2016) as template in a 10-fold dilution series (from 10 to 0.001 ng/µl). Singleplex qPCR assays were carried out twice, and for each assay, each dilution was replicated three times. A water control was included in triplicate in each assay. To detect any potential inhibition of amplification of *B. cinerea* DNA by *V. vinifera* DNA, duplex qPCR assays were performed with DNA of *B. cinerea* mixed with DNA of *V. vinifera*, following the approach described by Saito et al. (2013). In brief, 1 µl from each of the previously described DNA dilutions for *B. cinerea* was mixed with 1 µl of *V. vinifera* DNA (20 ng/µl), yielding a 10-fold dilution series from 1:2 to 1:20 000 w/w *B. cinerea*: *V. vinifera* DNA. The duplex qPCR assay was performed twice with three replicates for each dilution. Standard curves of both singleplex and duplex qPCR assays were produced by linear regression, and the coefficient of determination (R^2) was calculated. The amplification efficiency (E) of all assays was determined from the slope of the standard curves (Bustin et al. 2009). To allow comparisons among the results of different tests, all duplex qPCR plates contained a calibration DNA template in triplicate consisting of 1 ng/µl of *B. cinerea* DNA diluted in 20 ng/µl *V. vinifera* DNA (1:20 w/w *B. cinerea*:*V. vinifera* DNA).

Colonization coefficient. To quantify DNA of *B. cinerea* in bunch trash, known numbers of conidia or known weights of fresh mycelium were added to non-colonized bunch trash; the trash was obtained from the plants grown under isolation, and the absence of *B. cinerea* was confirmed by a Bc3 qPCR assay. Conidial suspensions (10 µl containing from 1 to 1,250 conidia/µl) or fresh mycelia (5.0, 1.0, 0.5, or 0.1 mg) were added to 0.1-g bunch trash samples in 2-ml microcentrifuge tubes. The total DNA from two biological replicates of each sample was then extracted as described earlier; non-colonized bunch trash without addition of *B. cinerea* was used as a negative control. Duplex qPCR assays were

performed twice for each experiment with two technical replicates of each template DNA. A water control and calibration DNA were included in each assay.

The quantification of DNA of *B. cinerea* in the presence of DNA of *V. vinifera* was expressed in terms of a colonization coefficient (CC), which was the ratio between DNA concentrations of *B. cinerea* and *V. vinifera*, corrected by a correction coefficient (ΔCC) (Gusberti et al. 2012). CC values were calculated as follows:

$$CC = DNA_{B.cinerea} / DNA_{V.vinifera} + \Delta CC \quad (1)$$

DNA amounts were obtained by transforming the quantification cycles (Cq) values of both targets (*B. cinerea* and *V. vinifera*) according to the standard curves obtained from the serial dilution assays (Table 4.2) as follows:

$$DNA\ (ng/\mu l) = 10^{[(Cq\ value - y\text{-}axis\ intercept)/slope]} \quad (2)$$

ΔCC was calculated as the difference between the average CC value of calibrator DNA calculated in the standard curve (CC_{St}= 5.15) and the average CC value of calibrator DNA of the assay (CC_A), (i.e., $\Delta CC = CC_{St} - CC_A$).

Evaluation of the qPCR method with inoculated bunch trash

Plant material. Bunch trash samples were collected in 2017 in a vineyard located in Castell'Arquato (CA) in the Emilia-Romagna region of Northern Italy (44°51′26.1″N 9°51′20.7″E, 400 m above sea level). The CA vineyard was planted to Merlot, which is highly susceptible to *B. cinerea* (Bisiach et al. 1996; Corvi and Tullio 1980). The vines were 10 years old and were trained using the Guyot system. The within and between-row spacings were 1.0 m and 2.3 m, respectively. The vineyard was managed following an integrated pest management (IPM) program, with between-row grass, branches pruned to 10-12 buds per cane, and no irrigation. Vines were not treated for control of *B. cinerea*. At full flowering (stage 65 of Lorenz et al. 1994), bunch trash was collected from the vines by gently shaking grape bunches inside paper bags. Bunch trash samples were transported to the laboratory and were immediately desiccated at 35-40°C for 72 h, and the dry weights determined. Samples were stored at room temperature.

Preparation of inoculum of *B. cinerea* and inoculation of bunch trash. Conidia of *B. cinerea* (isolate 213T) were obtained from 10-day-old cultures grown on PDA. The conidial suspensions were prepared by flooding the dishes with sterile-distilled water and gently scraping the agar surface with a sterile rod. The suspension was

filtered through two layers of autoclaved gauze and quantified using a hemocytometer. The inoculum concentration was adjusted to 10^5 conidia/ml.

The samples of bunch trash (0.1 g) collected in the vineyard were placed on autoclaved filter paper discs in Petri dishes (60 mm diameter), and inoculated with 1 ml of the conidial suspension of *B. cinerea* by using a micropipette. The samples were incubated at 20°C for 18 h in the dark to favor conidial germination and bunch trash colonization. The colonized samples were dried in a laminar flow hood at room temperature for 2h. Bunch trash samples with different degrees of colonization by *B. cinerea* (0, 25, 50, 75, and 100%) were obtained by mixing colonized and non-colonized bunch trash; for example, 75% colonization comprised 0.75 g of colonized bunch trash and 0.25 g of non-colonized bunch trash. Three replicate 1.0 g samples were prepared for each colonization level.

Colonization of inoculated bunch trash as determined by qPCR. In a first assay, genomic DNA was extracted from 0.1 g of two replicate samples for each of the five bunch trash colonization levels. The extracted DNA was quantified by the duplex qPCR assay described earlier. A water control and calibration DNA were included in each assay. The quantity of DNA of *B. cinerea* in the presence of DNA of *V. vinifera* was expressed as a CC value.

Colonization of inoculated bunch trash as determined by plating. In a second assay, colonization of inoculated bunch trash by *B. cinerea* was quantified for three replicate samples of each of the five colonization levels by randomly and individually plating 50 pieces (stamens, aborted flowers, aborted berries, calyptras, tendrils, or leaf fragments) on PDA in Petri dishes (diameter 90 mm). The dishes were incubated at 20°C with an 18 h photoperiod for 3 days. The dishes were examined using a stereomicroscope, and the colonization rate (CR) was expressed as the percentage of pieces with characteristic grayish sporulation indicating the growth of *B. cinerea*.

Sporulation potential on inoculated bunch trash as determined by incubation and spore enumeration. In a third assay, the sporulation potential (SP) of *B. cinerea* on inoculated bunch trash was determined for three replicate samples (0.05 g each) at each colonization level. The bunch trash was placed on a disc of autoclaved filter paper in Petri dishes (diameter 60 mm); sterile water (0.5 ml per dish) was used to moisten the filter paper to maintain a saturated atmosphere. The dishes were sealed with Parafilm and incubated at 20°C with an 18 h photoperiod to induce sporulation of *B. cinerea*. After 3 days of incubation, the bunch trash was suspended in 5 ml of sterile water in a 15-ml Falcon tube and mixed with a vortex apparatus for 10 seconds. Conidia of *B. cinerea* were counted using a hemocytometer and expressed as the number of conidia per g of dry bunch trash.

The bunch trash inoculation experiment and quantification *B. cinerea* by qPCR, plating and sporulation was performed three times.

Evaluation of the qPCR method with naturally inoculated bunch trash

Vineyards and treatments. Experiments were conducted in the CA vineyard and in two additional vineyards (designated MA and CO), located in Northern Italy in 2016 and 2017. The MA vineyard (44°41′57″N 12°19′66″E, at sea level) is located in Mandriole in the Emilia-Romagna region, and the CO vineyard (45°57′05″N 13°27′19″E, 1 m at sea level) is located in Cormons in the Friuli-Venezia Giulia region of Italy. The MA vineyard was planted with cv. Trebbiano Romagnolo, which were trained using the Casarsa system. Vines were 12 years old in 2016. The CO vineyard was planted with cv. Merlot, which were trained using the Guyot system. Vines were 7 years old in 2016. The within and between-row spacing in the MA and CO vineyard were 1.0 m and 3.0 m, and 0.8 m and 2.4 m, respectively. The MA vineyard was managed according to standard IPM practice in this region (Ministero delle politiche agricole alimentari, forestali e del turismo 2017), with between-rows was grass, vines pruned to 10 to 12 buds per cane, and emergency irrigation. The CO vineyard followed a conventional pest management strategy, with between-rows grass, vines long pruned, and irrigation. Like cv. Merlot in the CA and CO vineyards, cv. Trebbiano Romagnolo in the MA vineyard is highly sensitive to *B. cinerea* (Bisiach et al. 1996; Corvi and Tullio 1980).

In each of the three vineyards, fungicide treatments were applied to obtain a range of colonization of bunch trash by *B. cinerea*. There were two treatments: (i) NT, non-treated control; and (ii) T, fungicide applied at full flowering (stage 65). Treatments were arranged in a complete randomized block design with four replicate plots per treatment and with six plants per plot. The T treatment was a commercial mixture of fludioxonil (25%) and cyprodinil (37.5%) (Switch, Syngenta Crop Protection) applied at 0.8 g/l of water until run-off using a 15-L Elettroplus knapsack sprayer (Davide e Luigi Volpi S.p.a, Casalromano, Italy). Seven days after the treatment, bunch trash was collected from five random bunches per plot; these were combined to yield four replicate trash bunches per treatment.

Colonization of naturally inoculated bunch trash as determined by qPCR. Genomic DNA in the naturally inoculated bunch trash was extracted from each of the four replicate samples per treatment (0.1 g each) and was quantified by the duplex qPCR assay as described previously; there were two technical replicates of each template DNA. A water control and calibration DNA were included in each assay. The quantities of DNA of *B. cinerea* in the presence of DNA of *V. vinifera* were expressed as CC values.

Sporulation potential on naturally inoculated bunch trash as determined by incubation and spore enumeration. The sporulation potential (SP) of *B. cinerea* in the naturally inoculated bunch trash was determined for each of the four replicate samples per treatment by wrapping the bunch trash in three layers of sterile filter paper to which 5 ml of sterile water was added. The bunch trash in filter papers was sealed in polyethylene bags and incubated at 20°C for 5 days to induce sporulation in *B. cinerea*. Each sample of bunch trash was suspended in 15 ml of sterile water in a 50-ml falcon tube and vortexed. The conidia of *B. cinerea* were counted using a hemocytometer and the quantity expressed as the number of conidia per g of dry bunch trash.

Latent infection of naturally inoculated berries. The incidence of latent infection (ILI) of berries by *B. cinerea* was assessed in the three vineyards; 25 randomly selected, symptomless berries with the pedicel attached were collected at maturity (stage 89) in each replicate plot in both years. The berries were rinsed in tap water, surface sterilized by immersion for 1 min in a 30% sodium hypochlorite solution, and rinsed in sterile-distilled water for 1 min. Berries were positioned individually over a metal grid that was placed in a sterile metal box, the bottom of which was covered with wet, sterile paper. The boxes were sealed in plastic bags to maintain a saturated atmosphere and were incubated for 7 days at 25°C. The ILI was visually assessed as the percentage of berries showing typical sporulation of *B. cinerea*.

Data analysis

All statistical analyses were performed using SPSS (Version 24; IBM SPSS Statistics, IBM Corp., Armonk, NY). For experiments with inoculated bunch trash, regression analysis was used to investigate the relationships between the number of conidia of *B. cinerea* or the quantity of mycelium added and the colonization coefficient (CC), and between the CC and the colonization rate of bunch trash (CR) or the sporulation potential (SP) on bunch trash. Both linear and non-linear regression functions were used to explore these relationships: $Y = a + bX$; and $Y = Ymax/(1+\exp(a-bX))$, in which a and b are intercept and slope parameters, and Ymax is the maximum value of Y in the experiments. The coefficient of determination (R^2) was used to assess the strength of the relationship.

The data from the field experiment were subject to a factorial analysis of variance (ANOVA) to determine whether the quantity of DNA of *B. cinerea* in bunch trash, SP, and ILI were affected by main effects of year (2016 and 2017), vineyard (CA, CO, and MA), treatment (T and NT), and their interactions. Prior to the ANOVA, the SP and ILI values were transformed by natural logarithm and arcsine functions, respectively, to ensure homogeneity of variances.

Results

qPCR specificity and standard curves

The Bc3 probe/primer set did not amplify the purified DNA of non-target organisms but did amplify the purified DNA of *B. cinerea* (Table 4.1), demonstrating excellent specificity. In the singleplex qPCR reaction, the *B. cinerea* standard curve revealed a high reaction efficiency of 96% with a close relationship between the Cq values and the concentrations of DNA of *B. cinerea* obtained by dilution (Table 4.2). The Bc3 assay was able to amplify the lowest concentration of DNA of *B. cinerea* tested (0.001 ng/µl), demonstrating excellent sensitivity. In the duplex assay, the presence of grape DNA did not influence the sensitivity or coefficient of determination (R^2 value), whereas the reaction efficiency of the Bc3 set was slightly reduced (Table 4.2). Similar results were obtained for the *V. vinifera* standard curve. The Res assay was able to amplify the lowest concentration of DNA of *V. vinifera* tested (0.02 ng/µl).

Table 4.1. List of isolates screened during specificity tests of the real-time qPCR assay used to quantify *Botrytis cinerea* in grape bunch trash.

Genus and species	Isolate code	qPCR result[a]
Alternaria alternata	5	-
Alternaria sp.	23	-
Aspergillus flavus	4	-
Aspergillus niger	A1	-
Botrytis cinerea	213T and 351V	+
Erysiphe necator	FP 2017 and FP 2018[b]	-
Guignardia bidwellii	Q15 and C14	-
Monilia laxa	11	-
Penicillium sp.	2	-
Phomopsis viticola	Pho-1 and Pho-6	-
Plasmopara viticola	FP 2017 and FP 2018	-
Rhizopus sp.	26	-
Rhizopus stolonifer	MUCL38013	-
Sclerotinia sclerotiorum	22	-
Stemphylium sp.	14	-

[a] Minus signs indicates not amplified, and the plus sign indicates amplified.
[b] FP = field population and year of collection.

Table 4.2. Linear regression results and reaction efficiencies (E) for the relationship between serially diluted DNA concentrations (log transformed) of *Botrytis cinerea* and *Vitis vinifera* and corresponding Cq values obtained in singleplex and duplex qPCR assays.

qPCR assay	DNA template	Linear equation[a]	R^2 [b]	P value [c]	E (%)
Singleplex Bc3	*B. cinerea*	y = -3.42 x + 22.5	0.99	<0.001	96
Singleplex Res	*V. vinifera*	y = -3.37 x + 23.7	0.99	<0.001	98
Duplex Bc3/Res	*B. cinerea* + *V. vinifera*	y = -3.56 x + 24.0	0.99	<0.001	91

[a] In the equations, y refers to the Cq value, and x refers to the DNA concentration.
[b] R^2 = coefficient of determination of the regression.
[c] P value = indicates fit of the regression model.

Evaluation of the qPCR assay using inoculated bunch trash

The CC values, which represented the quantity of DNA of *B. cinerea* detected in the presence of DNA of *V. vinifera*, were proportional to the number of conidia of *B. cinerea* added to the bunch trash (Fig. 4.1A) and to the quantity of mycelium of *B. cinerea* added to the bunch trash (Fig. 4.1B); the coefficients of determination indicated a strong linear regressions between these variables (R^2 = 0.92 and 0.97, respectively). When the qPCR assay was used with bunch trash samples containing different proportions of non-colonized bunch trash and *B. cinerea*-colonized bunch trash, the CC values were strongly related to the percentage of bunch trash colonized by *B. cinerea* (Fig. 4.2A) (R^2 = 0.93).

For inoculated bunch trash, the relationship between the CC value and the sporulation potential (SP, as determined by incubation followed by microscopic counting of spores) and between the CC value and the colonization rate (CR, as determined by plating bunch trash pieces) was non-linear (Fig. 4.2B and 4.2C). As CC increased, both SP and CR increased, but the rate of increase of SP and CR declined while CC continues to increase resulting in a logistic relationship; the coefficients of determination indicated a strong relationship between these variables (R^2 = 0.92 and 0.97, respectively).

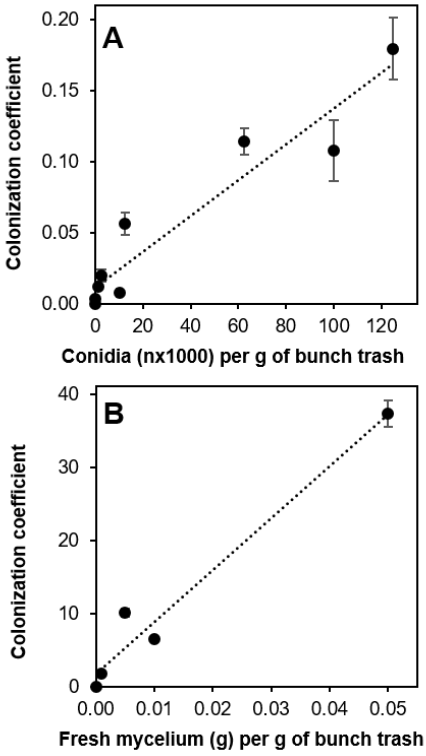

Figure 4.1. Relationship between the colonization coefficient (the ratio between DNA concentrations of *Botrytis cinerea* and *Vitis vinifera* as determined by the quantitative polymerase chain reaction assay) and **A,** the number of conidia added per gram of bunch trash, and **B,** the weight of fresh mycelium added to the bunch trash in inoculation experiments. Markers indicate means, whiskers indicate standard errors, and dotted lines indicate the linear relationships. In A, $Y=0.011+0.001X$ ($R^2=0.92$); and in B, $Y=1.75+710.74X$ ($R^2=0.97$).

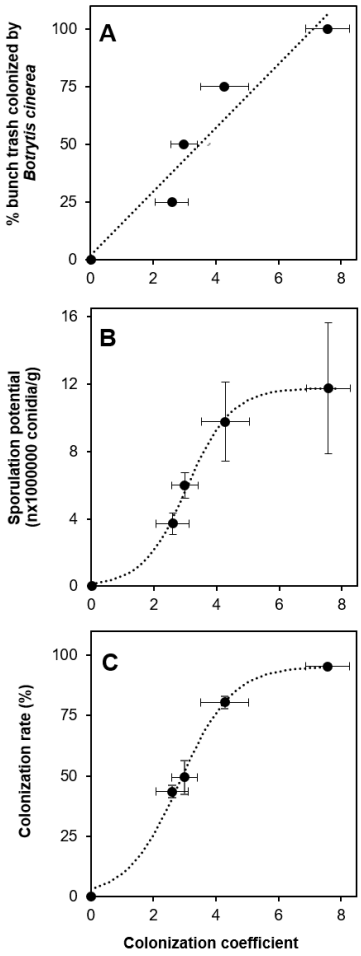

Figure 4.2. Relationship between the colonization coefficient (the ratio between DNA concentrations of *Botrytis cinerea* and *Vitis vinifera* as determined by the qPCR assay) of grape bunch trash and **A**, the proportion (%) of bunch trash that had been inoculated with and colonized by *B. cinerea*, **B**, the sporulation potential of the bunch trash (expressed as the number of conidia produced per gram of bunch trash as determined by incubating the bunch trash in humid chambers), and **C**, bunch trash colonization (expressed as the proportion (%) of bunch trash pieces that were colonized by *B. cinerea* as determined by plating the bunch trash on PDA). The grape bunch trash was inoculated with *B. cinerea* prior to being assayed for DNA of *B. cinerea*, sporulation potential, and colonization rate. Markers indicate means, whiskers indicate standard errors, and dotted lines indicate linear and non-linear relationships; in A: Y=*1.79+13.81*X (R^2=0.93); in B: Y=95.5/(1+exp(*3.38-1.19*X)) (R^2=0.99); in C: Y=1.17E+07/(1+exp(*4.31-1.41*X)) (R^2=0.99).

Evaluation of the qPCR assay using naturally colonized bunch trash

The application of fungicides to control *B. cinerea* at flowering significantly reduced the CC (colonization coefficient) (Fig. 4.3A), the SP (sporulation potential) (Fig. 4.3B), and the ILI (incidence of latent infection on berries) (Fig. 4.3C) ($P<0.001$ for all three variables; Table 4.3). Year had a significant effect on CC ($P\leq0.001$): the degree of colonization of bunch trash was greater in 2017 (CC=1.76±0.45) when compared with colonization in 2016 (CC=0.23±0.09). Main effects of year also affected SP ($P<0.0001$), but not ILI ($P=0.3$). The main effect of vineyard affected SP ($P=0.02$) and ILI ($P=<0.0001$), but not CC ($P=0.7$). In addition, SP was significantly influenced by the interaction year × vineyard, and ILI by the interactions year × vineyard and year × treatment. Thus, the fungicide treatment reduced CC, SP and ILI in all the vineyards, irrespective of the initial degree of colonization of bunch trash by *B. cinerea*, the sporulation potential of the bunch trash, or the incidence of latent infection of berries.

The interaction year × vineyard × treatment had no significant effect on CC ($P=0.6$), SP ($P=0.5$) or ILI ($P=0.8$), demonstrating that beyond the two-way interactions of main effects, there were no more complex associations in this study. These results indicate that the CC values reflected the reduction in sporulation of *B. cinerea* and the latent infection of berries caused by fungicide application at flowering. The qPCR method was able to detect differences between treated and nontreated plots in vineyards whether the colonization coefficient of bunch trash by *B. cinerea* was < 0.1 (Fig. 4.4A), < 3 (Fig. 4.4B), or < 5 (Fig. 4.4C).

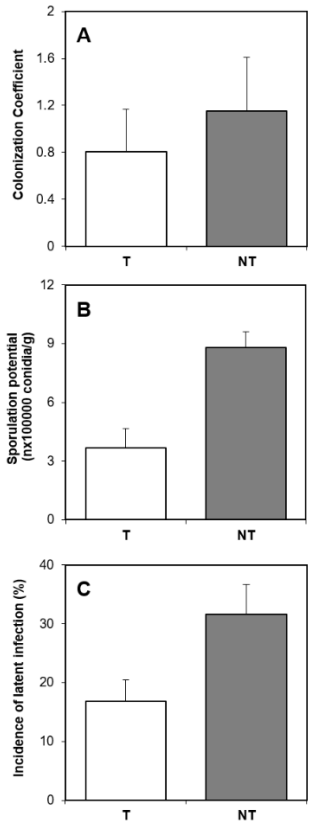

Figure 4.3. Effect of the application of fungicides (a mixture of fludioxonil (25%) and cyprodinil (37.5%) to control *Botrytis cinerea* at flowering on **A**, the colonization coefficient (the ratio between DNA concentrations of *B. cinerea* and *Vitis vinifera* in naturally colonized grape bunch trash as determined by the qPCR assay), **B**, the sporulation potential of bunch trash (expressed as the number of conidia produced per gram of naturally inoculated bunch trash after incubation in humid chambers), and **C**, the incidence of grape berries with latent infection (expressed as the percentage of berries showing the characteristic sporulation of *B. cinerea*). Bars indicate means of treated (T) and non-treated (NT) plots in three vineyards from two years data, and whiskers indicate standard errors (n=24 [3 vineyards, 2 years, 4 replicates]). In each panel, means are significantly different ($P<0.001$).

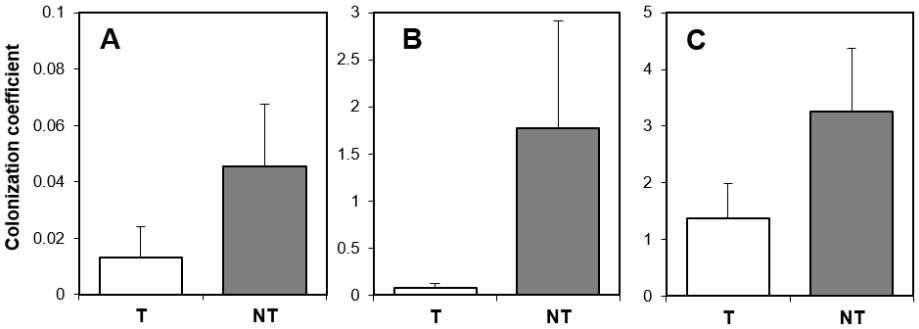

Figure 4.4. Ability of the qPCR assay to detect differences in the natural colonization by *Botrytis cinerea* of fungicide-treated (T) and non-fungicide-treated (NT) vines when the colonization coefficient of grape bunch trash was < 0.1 (**A**), < 3 (**B**), and < 5 (**C**). The data for A, B, and C were from the CA vineyard in 2017, the CO vineyard in 2016, and the MA vineyard in 2016, respectively. Fungicides (fludioxonil (25%) and cyprodinil (37.5%)) were applied at flowering. Bars indicate means of the colonization coefficient (the ratio between DNA concentrations of *B. cinerea* and *Vitis vinifera* in naturally inoculated grape bunch trash as determined by the qPCR assay), and whiskers indicate standard errors (n=4; based on 4 replicates). In all panels treatments are significantly different at P<0.001. The interaction year × vineyard × treatment was not significant (P=0.6).

Table 4.3. Results of the analysis of variance performed to explore main effects of fungicide treatment, vineyard and year, and main effect interactions on the quantity of DNA of *Botrytis cinerea* in bunch trash, the sporulation potential of bunch trash, and the incidence of grape berries with latent infection[a]

Main effects and interactions	d.f.[b]	Colonization coefficient		Sporulation potential		Incidence of latent infections	
		F value	P value	F value	P value	F value	P value
1. Year	1	17.4	<0.001	2309.3	<0.001	1.3	0.3
2. Vineyard	2	0.4	0.7	4.4	0.02	78.5	<0.001
3.Treatment	1	17.8	<0.001	50.6	<0.001	54.9	<0.001
1×2	2	1.7	0.2	21.8	<0.001	8.4	0.001
1×3	1	2.7	0.08	1.8	0.2	4.8	0.04
2×3	2	0.2	0.8	2.1	0.1	2.6	0.09
1×2×3	2	0.6	0.6	0.8	0.5	0.2	0.8

[a] The quantity of DNA of *B.cinerea* in bunch trash was defined as the colonization coefficient determined by the quantitative polymerase chain reaction assay,the sporulation potential of bunch trash was defined as the natural logarithm of the number of conidia produced per gram of naturally inoculated bunch trash after incubation in humid chambers, and the incidence of grape berries with latent infection was defined as the arcsin transformation of the percentage of berries showing the characteristic sporulation of *B. cinerea*. Samples were collected from a field experiment comparing grape bunches either treated with a commercial mixture of fludioxonil (25%) and cyprodinil (37.5%) (Switch; Syngenta Crop Protection) applied at 0.8 g/liter of water or not treated, with the experiment conducted in 2016 and 2017 in three vineyards in Italy.

[b] df = degrees of freedom.

Discussion

We evaluated a qPCR assay for the quantification of *B. cinerea* DNA in grape bunch trash. The qPCR assay was based on the procedure developed by Saito et al. (2013), with a few adaptations regarding the handling of plant material prior to DNA extraction and the qPCR reaction mixture and conditions. The qPCR assay was compared to traditional mycological techniques for quantifying *B. cinerea* based on colonization of, and sporulation on bunch trash. Our results indicate that the qPCR assay and the colonization coefficient (CC) calculation provide a sensitive and reliable method for quantifying colonization by *B. cinerea* of the trash materials (stamens, aborted flowers, aborted berries, calyptras, tendrils, and leaf pieces) remaining in grape bunches after flowering.

Colonized bunch trash serves as one of the primary sources of inoculum for the infection of ripening berries (Elmer and Michailides 2007; Holz et al. 2003; Nair et al. 1995). Thus, quantitative assessments of bunch trash colonization by *B. cinerea* is important for both research purposes and practical disease management, in order to make decisions regarding control of BBR. When the bunch trash has a low incidence of *B. cinerea*, subsequent development of BBR during berry ripening is likely to be low too (Keller et al. 2003; McClellan and Hewitt 1973; Pezet et al. 2003), and the number of fungicide applications can therefore be reduced (González-Domínguez et al. 2019, see Chapter 2). Visual assessment, plating on agar media, and microscope counts of spores have been commonly used to evaluate the colonization and the sporulation potential of *B. cinerea* in bunch trash (Abdelwahab and Younis 2012; Calvo-Garrido et al. 2014; Jaspers et al. 2013; Mundy et al. 2012); these traditional techniques are time-consuming and require expertise for the identification of *B. cinerea* colonies and/or conidia.

The results of Suarez et al. (2005) and those of our study indicate that the qPCR assay is highly specific to *B. cinerea*. We found that the Bc3 system amplified the DNA of different *B. cinerea* strains, including strains that belong to the transposon genotypes *transposa* (T) or *vacuma* (V) (Ciliberti et al. 2016), but did not amplify the DNA of phylogenetically related species (*B. fabae*, *Monilia laxa*, and *Sclerotinia sclerotiorum*). Also, the Bc3 system did not amplify the DNA of other common grapevine pathogens (*E. necator*, *Guignardia bidwellii*, *Phomopsis viticola*, and *Plasmopara viticola*) or of other fungal species frequently present in vineyards (*Alternaria* spp., *Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.).

In addition to its specificity, the qPCR assay is also sensitive because the targeted IGS region is a multi-copy gene (Bruns et al. 1991). The lowest DNA concentration of *B. cinerea* tested in this study (0.001 ng/µl) was amplified in both

the singleplex Bc3 assay and the duplex Bc3/Res assay in the presence of grape DNA, which is consistent with the results obtained by Saito et al. (2013) and Hill et al. (2014). Suarez et al. (2005) showed that the Bc3 assay is able to detect DNA concentrations as low as 20 fg/µl.

The results of the qPCR assay were used to calculate CC, i.e., the ratio of the pathogen and host DNA concentrations (Gusberti et al. 2012). In duplex qPCR analyses, researchers have described several methods for calculating the quantity of DNA of a pathogen in host tissue. To account for variation among samples and qPCR runs in terms of tissue weight, pipetting volumes, and efficiencies of DNA extraction and amplification, these methods account for amounts of host plant DNA in order to provide internal normalization. Sanzani et al. (2012), for example, normalized the DNA concentration of *B. cinerea* according to the quantity of host DNA by using a host DNA correction factor for each grape sample. Valsesia et al. (2005) developed the infection coefficient (IC), which is based on the ratio between Cq values of the pathogen and host generated by the qPCR assay. The IC approach was also used to determine the pathogen coefficient (PC) of *B. cinerea* in grape berries and receptacles (Hill et al. 2014; Saito et al. 2013). In a preliminary analysis, we found that the PC values based on the current data increased as the number of *B. cinerea* conidia or quantity of mycelium added to bunch trash increased. However, in the inoculation experiments, the PC values did not increase linearly as the level of colonization increased from 0 to 100% (data not shown). These preliminary results generally agree with those of Saito et al. (2013) and Hill et al. (2014), who found that the accuracy of PC decreased as the severity of BBR increased. For this reason, we used the CC rather than the PC in the current study.

The results of the qPCR assay were comparable to those obtained with the traditional methods used to estimate *B. cinerea* colonization of and sporulation on bunch trash. Therefore, the qPCR assay described in this work is a valuable alternative to the traditional methods. Traditional methods and qPCR require expertise in mycology and molecular biology, respectively. The traditional methods are time-consuming while the qPCR assay takes 3 to 4 hours. They also have limitations that potentially reduce their accuracy. Plating of trash pieces on agar can lead to the growth of other fast-growing fungal species that may result in lower estimates of the number of *B. cinerea* colonies; while the accurate enumeration of spores from incubated trash in humid chambers is dependent on the operator's expertise to correctly identify conidia. The qPCR, on the contrary, provides sensitive and specific results.

The validity of the qPCR assay was confirmed in the field using bunch trash naturally colonized with *B. cinerea* that had been treated or not treated with fungicides during flowering. In the field, the CC values were consistent with the reduction of the sporulation potential caused by fungicide treatment and revealed differences between fungicide-treated plants and non-treated plants under different environmental conditions, even in situations where the incidence of colonization of bunch trash by *B. cinerea* was very low. The CC values of bunch trash were also consistent with the reduction in the incidence of latent infection of berries caused by fungicide application at flowering. Although the latter result requires confirmation, it suggests that when the colonization of bunch trash is low, the incidence of latent infection is also low due to unfavorable conditions for reproduction of *B. cinerea* during flowering.

In conclusion, the qPCR methodology described here is a sensitive, specific and reliable tool for quantifying *B. cinerea* in bunch trash in vineyards. The qPCR assay can be used as an alternative to traditional methods for the quantification of *B. cinerea* during the early-season period (as an indicator of inoculum potential) and thus BBR severity at harvest; it can also be used as a tool in other epidemiological studies, and to determine the effect of disease management methods on the reduction of inoculum of *B. cinerea*.

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Chapter 5

Chapter 5

Reduction of *Botrytis cinerea* colonization of and sporulation on bunch trash[1]

Abstract

Botrytis bunch rot (BBR) of grapevine, caused by *Botrytis cinerea*, is commonly managed by fungicide sprays at flowering (A), pre-bunch closure (B), veraison (C), and before harvest (D). Applications at A, B, and C are recommended to reduce *B. cinerea* colonization of bunch trash and the production of conidia during berry ripening. The effects of these applications were previously evaluated as reductions in BBR severity at harvest rather than as reductions in bunch trash colonization and sporulation by *B. cinerea*. The current study investigated the effects of fungicides (FUN, a commercial mixture of fludioxonil and cyprodonil), biological control agents (BCA, *Aureobasidium pullulans* and *Trichoderma atroviride*), and botanicals (BOT, a commercial mixture of eugenol, geraniol, and thymol) applied at different timings (A, B, C, or ABC) compared to a non-treated control (NT) on *B. cinerea* bunch trash colonization and sporulation in vineyards. The ability of B*. cinerea* to colonize the bunch trash (as indicated by *B. cinerea* DNA content) and to sporulate (as indicated by the number of conidia produced under optimal laboratory conditions) was highly variable, and this variability was higher between years (2015 to 2018) than among the three vineyards and three sampling times (i.e., 1 week after applications at A, B, and C). *Botrytis cinerea* sporulation on bunch trash was significantly lower in plots treated with FUN than in NT in only 3 of 18 cases (3 vineyards × 2 years × 3 sampling times). FUN applications, however, significantly reduced *B. cinerea* colonization of bunch trash compared to NT; for colonization, BCA efficacy was similar to that of FUN, but BOT efficacy was variable. For all products, colonization reduction was the same with application at A vs. ABC, meaning that the effect of an early season application lasted from flowering to 1 week after veraison. These results indicate that the early season control of *B. cinerea* is important to reduce the saprophytic colonization of bunch trash, especially when the risk of BBR is high.

[1] Fedele G, González-Domínguez E, Si Ammour M, Languasco L, Rossi V, 2020. Plant Disease, DOI: 10.1094/PDIS-08-19-1593-RE

Introduction

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) attacks many economically important crops including grapevine (*Vitis vinifera* L.), causing Botrytis bunch rot (BBR) (Elmer and Michailides 2007). *Botrytis cinerea* develops and grows as a plant pathogen, and as a saprophyte on various organs of host plants (Jarvis 1977; van Kan 2006). The fungus can produce a large number of conidia on grape bunch, bunch and leaf trash, and rotted berries under a wide range of environmental conditions (Ciliberti et al. 2016; Mundy et al. 2012; Nair et al. 1995). The fungus has multiple infection pathways (Elmer and Michailides 2007), with infection mainly occurring from flowering to young cluster, and after veraison. In the first period, conidia germinate and infect the flower style and ovules (pathway I), the stamens or petals (pathway IIa), or the fruit pedicel (pathway IIb) (Elmer and Michailides 2007). Infections can cause blossom blight and latent infection of berries; under suitable environmental conditions, latent infections result in rotted berries after veraison (Holz et al. 2007; Keller et al. 2003; McClellan and Hewitt 1973). Grape inflorescences are more susceptible at flowering, fruit swelling, or “berry groat-size” than at earlier growth stages (Ciliberti et al. 2015). During flowering, the pathogen saprophytically colonizes the bunch trash (the dead stamens, aborted flowers, aborted berries, calyptras, and tendrils) retained within the developing bunches, then occurring conidial germination and extensive colonization of floral debris in grape bunches (pathway III, Elmer and Michailides 2007). Under favorable conditions, the colonized bunch trash produces conidia that can infect the ripening berries as part of pathway IV with conidial accumulation within the developing bunch (Elmer and Michailides 2007). Ripening berries can also be infected by airborne conidia (pathway Va), and through contact with the aerial mycelium produced on adjacent moldy berries (pathway Vb, berry-to-berry infection) (González-Domínguez et al. 2015).

BBR control is currently based on the application of fungicides at four grape growth stages (GS): A, end of flowering (GS69; Lorenz et al. 1995); B, pre-bunch closure (GS77); C, veraison (GS83); and D, before harvest (before GS89) (Broome et al. 1995; Bulit et al. 1970). The early season applications (A and B) are aimed at i) reducing conidial germination and infection of flowers, ii) preventing latent infections of berries, and iii) disinfesting the bunch trash. The later-season applications (i.e., the applications from veraison until harvest; C and D) are aimed not only at preventing berry infection during ripening but also at disinfesting bunch trash in order to reduce the inoculum load (Baldacci et al. 1962; Calvo-Garrido et al. 2014a). Bunch trash colonized by *B. cinerea* is therefore considered an

important source of inoculum for infection from flowering until ripening (Calvo-Garrido et al. 2014a; Holz et al. 2003; Nair et al. 1995; Viret et al. 2004), and the incidence of *B. cinerea* in bunch trash is associated with the severity of BBR at harvest (McClellan and Hewitt 1973; Keller et al. 2003; Seyb et al. 2000).

The Directive 128/2009/EC on the Sustainable Use of Pesticides makes it mandatory for the EU Member States to use pest control strategies based on low pesticide input. The interest in sustainable BBR control is a direct consequence of the negative public perception regarding the effects of chemicals on human health and the environment (Alavanja et al. 2004; Epstein 2014), and of the development of *B. cinerea* populations with resistance to chemical fungicides (Fernández-Ortuño et al. 2016; Leroux 2007). Thus, biological control agents and botanicals are considered alternatives or complementary to chemical fungicides for the control of BBR in vineyards (Calvo-Garrido et al. 2019; Calvo-Garrido et al. 2014b; O'Neill et al. 1996; Pertot et al. 2017; Rotolo et al. 2018; Ştefan et al. 2015; Walter et al. 2001). Both biological control agents and botanicals have been mainly studied for their efficacy in reducing BBR on ripening bunches (Calvo-Garrido et al. 2019; O'Neill et al. 1996; Pertot et al. 2017; Rotolo et al. 2018; Ştefan et al. 2015; Walter et al. 2001), but their ability to reduce the colonization of bunch trash and the subsequent production of conidia by *B. cinerea* has seldom been studied. In a 2-year field experiment, Calvo-Garrido et al. (2014b) observed that the early season application of *Candida sake*, *Ulocladium oudemansii*, or chitosan reduced the mycelial growth and sporulation of *B. cinerea* on bunch trash. No information exists, however, on the effect of biological control agents or botanicals in the late season on *B. cinerea* colonization and sporulation on bunch trash.

The general aim of this research was to investigate the use of fungicides, biological control agents, and botanicals for bunch trash disinfestation in vineyards. Two experiments were conducted with the following objectives: i) evaluate the effect of different timings of fungicide applications (A, B, C, or ABC) in reducing the sporulation of *B. cinerea* on bunch trash under different levels of disease pressure; and ii) compare the effectiveness of BBR control products (FUN, BCA, and BOT) applied at different timings (A, B, C, or ABC) in reducing the saprophytic colonization of bunch trash by *B. cinerea*.

Materials and methods

Sporulation of *B. cinerea* on bunch trash as affected by the timing of fungicide application

Vineyards and treatments. Experiment 1 was conducted in 2015 and 2016 in three experimental vineyards in Northern Italy. The CA vineyard is located at Castell'Arquato (44°51′26.1″N 9°51′20.7″E, 400 m a.s.l.) in the Emilia-Romagna region; the MA vineyard is located at Mandriole (44°41′57″N 12°19′66″E, 0 m a.s.l.), also in the Emilia-Romagna region; and the CO vineyard is located at Cormons (45°57′05″N 13°27′19″E, 1 m a.s.l.) in the Friuli-Venezia Giulia region. The CA and CO vineyards were planted with cv. Merlot, and the MA vineyard was planted with cv. Trebbiano Romagnolo; both Merlot and Trebbiano Romagnolo are highly susceptible to *B. cinerea* (Bisiach et al. 1996; Corvi and Tullio 1980). The vines in the CA vineyard were 8 years old in 2015 and were trained using a Guyot system; the within- and between-row spacing were 1.0 and 2.3 m, respectively. The vines in the MA vineyard were 11 years old in 2015 and were trained using the Casarsa system; the within- and between-row spacing were 1.0 m and 3.0 m, respectively. The vines in the CO vineyard were 6 years old in 2015 and were trained using the Guyot system; the within- and between-row spacing were 0.8 m and 2.4 m, respectively. Powdery and downy mildews were controlled according to an integrated pest management (IPM) program (Rossi et al. 2012) in the CA and MA vineyards but by a conventional disease management program in the CO vineyard. In all three vineyards, the fungicides applied were ineffective against *B. cinerea*. In each vineyard, hourly data of temperature, relative humidity, wetness duration, and rainfall were recorded by an automated weather station (iMeteos, Pessl Instruments GmbH, Weiz, Austria) located < 1 km from the experimental plot. Growth stages of vines (GS) were assessed weekly in the vineyards according to the scale of Lorenz et al. (1995).

Assessment of sporulation potential of *B. cinerea*. In all vineyards, four timings of fungicide (FUN) application were compared: A (full flowering; GS65 of Lorenz et al. 1995), B (pre-bunch closure; GS77), C (veraison, GS83), or ABC. A non-treated control (NT) was also included. The four applications and control were arranged in a completely randomized design, with four replicates and seven plants per plot. A commercial mixture of fludioxonil (25%) and cyprodonil (37.5%) (Switch; Syngenta Italia S.p.A., Milano, Italy) at the label dose (800 g/ha) was applied until run-off using a 15-L Elettroplus knapsack sprayer (Davide e Luigi Volpi S.p.A., Casalromano, Italy). Dates of application are indicated in Figure 5.1. Seven days after each application, bunch trash samples were collected from five randomly selected bunches in each replicate plot. This was done by gently shaking the five bunches inside one paper bag. Bunch trash samples were immediately transported

to the laboratory and dried at 35-40°C for 72 h before the dry weight was determined. The total bunch trash of each replicate was then packed in polyethylene bags containing three pieces of wet filter paper to maintain 100% relative humidity, and were incubated at 20°C for 5 days in darkness. The bunch trash was subsequently suspended in 15 ml of sterile water in a 50-ml centrifuge tube and was mixed with a vortex apparatus for 1 min. Finally, *B. cinerea* conidia were counted with the aid of a hemocytometer (Bürker, HBG) using a dissecting microscope and expressed as the number of conidia per g of dry weight.

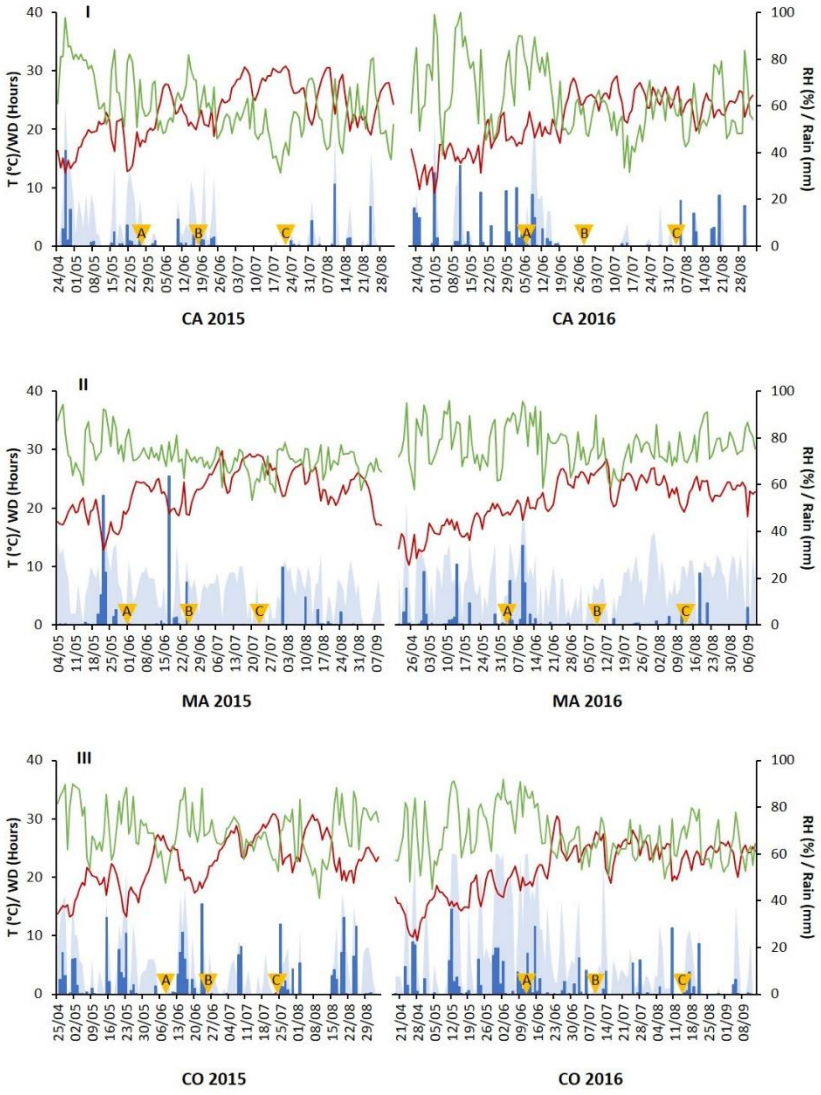

Figure 5.1. Weather conditions in the Castell'Arquato (CA), Mandriole (MA), and Cormons (CO) vineyards in 2015 and 2016 (experiment 1). Daily values of temperature (T; red line; in degrees Celsius), RH (green line; in percentage), rain (blue bars; in millimeters), and wetness duration (WD; light blue area; in hours) between the grape growth stage inflorescences clearly visible and veraison (GS53 and GS83 of Lorenz et al. 1995, respectively). The yellow triangles indicate the timing of application of a fungicide for controlling *Botrytis cinerea*: A (full flowering; GS65), B (prebunch closure; GS77), and C (veraison; GS83).

Colonization of bunch trash by *B. cinerea* as affected by product and timing of application

Vineyards and treatments. Experiment 2 was carried out in 2017 and 2018 in the CA vineyard. Three products and four timings were arranged in a split-plot design, with four replicate plots (six plants per plot) for each combination of timing (main plot) × product. Timings were the same as in experiment 1 (A, B, C, or ABC); a non-treated control (NT) was also included. The following products were compared: i) FUN, a commercial mixture of fludioxonil (25%) and cyprodonil (37.5%) (Switch; Syngenta Italia S.p.A., Milano, Italy) at the label dose (800 g/ha); ii) BOT, a commercial mixture of eugenol (3.2%), geraniol (6.4%), and thymol (6.4%) (3LOGY; Sipcam Italia S.p.A., Pero, Italy) at the label dose (4000 ml/ha); iii) BCA, *Aureobasidium pullulans* (Botector; Manica S.p.A., Rovereto, Italy) only at A and C, and *Trichoderma atroviride* (Vintec; Belchim Crop Protection Italia S.p.A., Rozzano, Italy) only at B (Pertot et al. 2017); the BCA products were sprayed at the label dose (400 and 1000 g/ha, respectively). All products were applied until run-off using a 15-L Elettroplus knapsack sprayer (Davide e Luigi Volpi S.p.A., Casalromano, Italy). Dates of application are indicated in Figure 5.2. Seven days after each application, bunch trash samples were collected by gently shaking five randomly collected bunches per plot inside paper bags. Bunch trash samples were immediately transported to the laboratory, dried at 35-40°C for 72 h, and weighed. The dry samples were then assessed for *B. cinerea* colonization rate as described by Si Ammour et al. (2019, see Chapter 4), and as summarized below.

DNA extraction. Genomic DNA was extracted from 100-mg (dry weight) samples of bunch trash (four replicate samples for each combination of product and timing). Each bunch trash sample was placed in a 2-ml microcentrifuge tube containing 100 mg of glass sand (425-600 µm diameter), two glass beads (5 mm diameter), and 500 µl of cetyl trimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM ethylenediaminetetraacetic acid [EDTA], 1.4 M NaCl, and 1% polyvinylpyrrolidone [PVP]). The tubes were placed in a Mixer Mill MM200 (Retsch GmbH, 93 Haan, Germany) for 1 min at 30 cycles/s. The mixture was then vigorously mixed with a vortex apparatus and heated for 15 min at 65°C. A 500-µl volume of chloroform-isoamyl alcohol (24:1, v:v) was added. After further vigorous mixing, the tubes were centrifuged at 12000 rpm for 10 min, and the supernatant was transferred to a new microcentrifuge tube. The chloroform-isoamyl alcohol purification were repeated. The supernatant was transferred to a new microcentrifuge tube, and a 65°C solution of 10% CTAB with 0.7 M NaCl was added at a rate of 1:10 (v/v). A third chloroform-isoamyl alcohol purification and centrifugation was performed, and the resulting supernatant was

transferred to a new microcentrifuge tube, to which was added an equal volume of cold (approximately 0°C) isopropanol and a 10% volume of 3 M sodium acetate; this was followed by centrifugation at 12000 rpm for 5 min at 4°C. The pellet was washed with 70% (v/v) ethanol, air dried, and resuspended in 100 µl of sterile-distilled water. The yield and purity of the extracted DNA were determined using a NanoDrop™2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The extracts were adjusted to 20 ng/µl of DNA.

Real-time PCR. A duplex qPCR assay was used to assess the colonization of bunch trash by *B. cinerea* as described by Si Ammour et al. (2019, see Chapter 4). The primers/hydrolysis probe set Bc3 was used to amplify the intergenic spacer region (IGS) of the nuclear ribosomal DNA of *B. cinerea* (Suarez et al. 2005). To normalize the quantification of *B. cinerea* DNA in the bunch trash, the primers/hydrolysis probe set Res was used to amplify the *V. vinifera* resveratrol synthase gene I (Valsesia et al. 2005). The duplex reaction mixtures contained 1x QuantiTect Multiplex PCR Kit, 150 nM of the *V. vinifera* probe ResP, 150 nM of the *B. cinerea* probe Bc3P, 100 nM of each *V. vinifera* primer (Res F/R), 500 nM of each *B. cinerea* primer (Bc3F/R), and 2 µl of DNA template in a final volume of 10 µl. The assay was performed using an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA) with an initial incubation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Two technical replicates of each template DNA were sequentially quantified by the duplex qPCR assay. A water control was included in each assay. DNA amounts (in ng/µl) were obtained by transforming the quantification cycles (Cq) of both targets (*B. cinerea* and *V. vinifera*) according to the standard curves obtained from the serial dilution assays performed by Si Ammour et al. (2019, see Chapter 4). The quantity of *B. cinerea* DNA in a sample was expressed as a colonization coefficient (CC), which was the ratio of *B. cinerea* DNA concentration to *V. vinifera* DNA concentration (Gusberti et al. 2012).

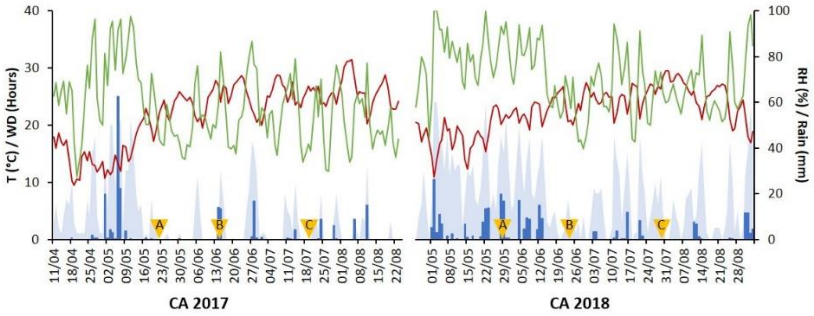

Figure 5.2. Weather conditions in the vineyard of Castell'Arquato in 2017 and 2018 (experiment 2). Daily values of temperature (red line, T in °C), relative humidity (green line, RH in %), rain (blue bars, in mm), and wetness duration (light blue area, WD in hours) between the grape growth stage "inflorescences clearly visible" and "veraison" (GS53 and GS83 of Lorenz et al. 1995, respectively). The yellow triangles indicate the timing of application of products for controlling *Botrytis cinerea*: A (full flowering; GS65), B (pre-bunch closure; GS77), or C (veraison; GS83).

Data analyses

Data were analyzed with R software (v 3.6.0; R core team, 2019). The dataset analyzed for experiment 1 consisted of the number of *B. cinerea* conidia/g of bunch trash assessed in three vineyards (CA, MA, and CO), in two years (2015 and 2016), in plots treated with FUN and NT, at three sampling times (1 week after fungicide application at A, B, and C). The dataset analyzed for experiment 2 consisted of the colonization coefficient (CC) of *B. cinerea* in bunch trash assessed in the CA vineyard, in two years (2107 and 2018), and in plots treated with different products (FUN, BOT, BCA, or NT) and at different timings (A, B, C, or ABC), on the 7th day after veraison (i.e., 1 week after application at C).

In a preliminary analysis conducted with the non-parametric Kruskal-Wallis test (by using the function *kruskal.test*), the numbers of conidia (experiment 1) and the CC (experiment 2) in the non-treated bunch trash strongly and significantly ($P<0.001$) differed between the 2 years of each experiment. Therefore, the data were analyzed separately for the two years. Generalized linear models (GLMs) were fit to the data by using the function *glm* of the 'lme4' package (Bates et al. 2011). In the first dataset, timings of applications were considered as fixed factors, alone (models 1, 3, 5, 7, 9, and 11 in Table 5.1) or in an interaction with vineyard (models 2, 4, 6, 8, 10, and 12 in Table 5.1). In these models, the *log* link function (transformation) was used for the number of *B. cinerea* conidia/g of bunch trash, and the quasi-Poisson distribution of errors was selected to compensate for over-

dispersion (Crawley 2013) due to a residual deviance (*D*) that was higher than the degrees of freedom (*df*). The best model was selected by comparing the models with an F test with the function *anova* (e.g., between models 1 and 2) (Crawley 2013). In the second dataset, products were considered as fixed factors, either alone (models 1 and 4 in Table 5.2), with the inclusion of timing of applications (models 2 and 5 in Table 5.2), or as an interaction with timing of applications (models 3 and 6 in Table 5.2). In these models, the Binomial distribution and the *logit* link function were used. The best model was selected based on the reduction of Akaike's Information Criterion (AIC), considering that a reduction >2 indicates better model performance (Burnham and Anderson 2002). The effect of each factor in the selected model was tested by a *chi.test* with the function *anova*. For all selected models, the assumptions of normality and homogeneity of variance (homoscedasticity) were confirmed based on the visual examination of the standardized deviance residuals against the theoretical quantiles and against the predicted values (Crawley 2013; Madden et al. 2000).

In the first dataset, differences between each timing of FUN application and the NT control were tested by a contrast analysis; these pairwise combinations were tested by using the *glht* function of the 'multcomp' package (Hothorn et al. 2008). In the second dataset, the difference between FUN and NT was used as the intercept of the model, and its probability was calculated. Afterwards, differences between BOT and FUN, and between BCA and FUN were tested based on the GLM estimates; differences between ABC and each timing of application (i.e., A, B, and C) were also tested.

For both datasets, data are shown as estimated efficacy and the 95% confidence interval (calculated by using the inverse of the link function). Efficacy (E) was calculated as E=(NT-T)/NT, where NT is the value of the non-treated control and T is the value in any specific application (e.g., the number of *B. cinerea* conidia in plots treated with FUN at timing A, in vineyard CO, in 2016). In a further analysis, the outcome of FUN application in experiment 1 was considered as 0 (i.e., no significant reduction of sporulation compared to NT) or 1 (i.e., significant reduction). The relationship between this outcome and the number of conidia/g in the NT was assessed by running a binary logistic function, using the *glm* function (with binomial distribution and *logit* link function), in the form $Y = 1/(1 + \exp(a - bX))$, in which *a* and *b* are intercept and slope parameters. To evaluate the effect of the sporulation level on the significant reduction following a FUN application, a *chi.test* of this model was conducted by using the function *anova*.

Table 5.1. General linear models (GLMs) used to study the effect of timing of application of a fungicide (FUN) in different vineyards on the sporulation potential of *Botrytis cinerea* on bunch trash (experiment 1).

Year	Sampling time[a]	Model[b]	Factor[c]	df	F[d]	P
2015	A	1	*Timing*[e]	70		
		2	*Timing*× *Vineyard*[f]	66	27.00	<0.001
	B	3	*Timing*	140		
		4	*Timing*× *Vineyard*	132	15.91	<0.001
	C	5	*Timing*	166		
		6	*Timing*× *Vineyard*	156	24.83	<0.001
2016	A	7	*Timing*	70		
		8	*Timing*× *Vineyard*	66	5.23	<0.001
	B	9	*Timing*	137		
		10	*Timing*× *Vineyard*	129	13.80	<0.001
	C	11	*Timing*	175		
		12	*Timing*× *Vineyard*	165	16.82	<0.001

[a] 1 week after application at A (full flowering; GS65 of Lorenz et al. 1995), B (pre-bunch closure; GS77), or C (veraison; GS83).
[b] Different GLMs were fit for each year, all with quasi-poisson distribution and a *log* link function.
[c] Timing of application was considered a fixed factor, alone, or as interaction with vineyard.
[d] F test and the associated probability (P) when comparing models with the same dataset.
[e] Timings of FUN applications were A (full flowering; GS65 of Lorenz et al. 1995), B (pre-bunch closure; GS77), C (veraison; GS83), or ABC; timing was considered a fixed factor, alone or as interaction with vineyard. FUN was a commercial mixture of fludioxonil (25%) and cyprodonil (37.5%) (Switch; Syngenta Italia S.p.A., Milano, Italy) at the label dose (800 g/ha).
[f] Vineyards were CA (Castell'Arquato), MA (Mandriole), and CO (Cormons).

Table 5.2. Summary of the generalized linear models (GLMs) fitted to the data to investigate the efficacy of different products and timings of applications in reducing bunch trash colonization by *Botrytis cinerea* at the end of the season (experiment 2).

Year	Model[a]	Factors[b]	Residual deviance[e]	df[f]	Null deviance	df	AIC[g]
	1	*Product*[c]	66.53	76	84.65	78	94.65
2017	2	*Product*+ *Timing*[d]	61.32	73	84.65	78	91.52
	3	*Product*× *Timing*	55.09	67	84.65	78	99.40
	4	*Product*	44.57	77	46.62	79	98.69
2018	5	*Product*+ *Timing*	42.39	74	46.62	79	101.58
	6	*Product*× *Timing*	40.90	68	46.62	79	113.07

[a] Different GLMs were run for each year, all with Binomial distribution and a *logit* link function.
[b] Product was considered a fixed factor, alone, with timing of application, or as interaction of both factors.
[c] Products were: i) FUN, a commercial mixture of fludioxonil (25%) and cyprodonil (37.5%) (Switch; Syngenta Italia S.p.A., Milano, Italy) at the label dose (800 g/ha); ii) BOT, a commercial mixture of eugenol (3.2%), geraniol (6.4%), and thymol (6.4%) (3LOGY; Sipcam Italia S.p.A., Pero, Italy) at the label dose (4000 ml/ha); and iii) BCA, *A. pullulans* (Botector; Manica S.p.A., Rovereto, Italy) at A and C, and *T. atroviride* (Vintec; Belchim Crop Protection Italia S.p.A., Rozzano, Italy) at B (Pertot et al. 2017). Both BCA products were sprayed at the label dose (400 g/ha and 1000 g/ha, respectively).
[d] Timings of applications were A (full flowering; GS65 of Lorenz et al. 1995), B (pre-bunch closure; GS77), and C (veraison; GS83).
[e] Residual deviance: –2 times the likelihood for the fitted model minus the likelihood for the saturated model (in which the fitted values equal the observation).
[f] Residual degrees of freedom.
[g] AIC: Akaike's information criterion.

Results

Sporulation of *B. cinerea* on bunch trash as affected by the timing of fungicide application

Weather conditions at the three vineyards and in the two years were different (Fig. 5.1). At CA, the period between "inflorescences clearly visible" and "veraison", GS53 and GS83, respectively, was 102 and 109 days long in 2015 and 2016, respectively. The weather was rainy and moist between the GS53 and full flowering (GS65) in both years (100 mm of rain and 200 h of wetness in 2015, 200 mm and 108 h in 2016); in the following period, both rain and hours of wetness were lower (Fig. 5.1I). At MA, the experimental period was 80 days in 2015 and 113 in 2016. In both years, more than 200 mm of rain and more than 400 h of wetness were registered between inflorescence development (GS53) and pre-bunch closure (GS77); the later period was drier until GS83 (Fig.5.1II). At CO, 89 and 120 days passed between GS53 and GS83 in 2015 and 2016, respectively. In the period between GS53 and GS77, abundant rain fell (more than 300 mm in both years), and 355 h of wetness and > 700 h of wetness were registered in 2015 and 2016, respectively. Differences were found between years in the period from GS77 to GS83, with only 37.9 mm of rain and 70 h of wetness in 2015 and 74.0 mm of rain and 145 h of wetness in 2016 (Fig. 5.1III).

The average number of *B. cinerea* conidia/g in the NT bunch trash was significantly higher in 2015 than in 2016 ($4.34\pm0.94\times10^5$ vs. $9.67\pm1.51\times10^2$) ($P<0.001$), indicating a higher level of sporulation potential in 2015 than in 2016. Differences were also observed among vineyards in the same year and among sampling times in the same vineyard (Table 5.3). In vineyard CO in 2015 and 2016, for example, the sporulation potential increased over time. In vineyard CA in 2015, sporulation was high at GS65, very low at GS77, and very high at GS83; in other vineyards, sporulation was higher at GS77 than at GS65 or GS83 (Table 5.3).

In each year, the number of *B. cinerea* conidia produced on bunch trash collected 1 week after flowering, pre-bunch closure, or veraison was affected by the interaction between vineyard and timing of fungicide application ($P<0.001$). Models considering the interaction between timing and vineyard were selected based on their significance when compared with those that considered only the timing of fungicide applications (models 2, 4, 6, 8, 10, and 12, Table 5.1); the assumptions of independence of errors, normality, and homoscedasticity of the residuals were confirmed (*not shown*). Based on these models, the sporulation of *B. cinerea* on bunch trash was significantly lower for plots treated with FUN at different timings than for NT plots for only the following three cases among the 18 combinations of sampling times, vineyard, and year. The first case refers to the sporulation of bunch trash samples collected 1 week after flowering in vineyard

MA, in 2015 (Table 5.3), which was reduced by FUN applied at A compared to NT (P=0.030), with an estimated efficacy ranging from 0.53 to 0.68 (Fig. 5.3I). The second case refers to the sporulation of bunch trash samples collected 1 week after veraison in vineyard CA, in 2015 (Table 5.3), which was reduced by FUN application either at A, B, C or ABC (P<0.001), with estimated efficacy ranging from 0.78 to 0.94 (Fig. 5.3II). The third case refers to the sporulation of bunch trash samples collected 1 week after veraison in vineyard CO, in 2016 (Table 5.3), which was reduced by FUN application either at A, C, or ABC (P<0.001), but not at B (P=0.515), with estimated efficacy ranging between 0.55 and 0.70 (Fig. 5.3III). Sporulation on the NT bunch trash was very low for the third case, intermediate for the first case, and high for the second case (Table 5.3).

When all of the sporulation data in Table 5.3 were combined with the outcomes of FUN applications (considered as: 0 = no significant reduction of sporulation; or 1 = significant reduction) in a binary logistic function (with P=0.051), the probability to obtain a reduction in the sporulation following a FUN application increased as the sporulation potential of bunch trash increased. The estimated parameters of the logistic equations were -2.294 (±0.852) for the intercept (*a*) and 2.201 (±1.994)×10^{-6} for the explanatory variable (i.e., conidia/g of bunch trash; *b*); therefore, probability=0.5 when the sporulation potential is 10.423×10^{5} conidia/g. This means that when the sporulation potential of *B. cinerea* on bunch trash was lower than this value, the probability that a fungicide application was effective was < 0.5.

Table 5.3. Number of *Botrytis cinerea* conidia produced per g of bunch trash collected from plots that were not treated with fungicide (NT) in three vineyards in 2015 and 2016 (experiment 1).

Year	Vineyard[a]	Sampling time:1 week after		
		Flowering (GS65)	**Pre-bunch closure (GS77)**	**Veraison (GS83)**
2015	CA	6.03 (4.84-7.51) [b]×10^5	0.40 (0.13-1.26)×10^5	25.84 (20.55-32.45)×10^5
	MA	2.72 (1.96-3.77)×10^5	3.37 (2.27-5.01)×10^5	0.69 (0.20-2.31)×10^5
	CO	1.03 (0.60-1.75)×10^5	1.25 (0.66-2.40)×10^5	3.12 (1.76-5.51)×10^5
2016	CA	0.54 (0.39-0.75)×10^3	2.12 (1.44-3.12)×10^3	0.26 (0.15-0.44)×10^3
	MA	0.42 (0.29-0.60)×10^3	2.82 (2.02-3.94)×10^3	0.21 (0.12-0.38)×10^3
	CO	0.12 (0.06-0.24)×10^3	0.72 (0.37-1.40)×10^3	1.54 (1.24-1.92)×10^3

[a] CA, Castell'Arquato; MA, Mandriole; CO, Cormons.
[b] Values and confidence intervals (95%) of the numbers of conidia produced by *Botrytis cinerea* on bunch trash after incubation at 20°C, 100% RH, for 5 days, estimated by transforming the parameters of the generalized linear models (GLMs) on their response scale (see Table 5.2 for the GLMs fit).

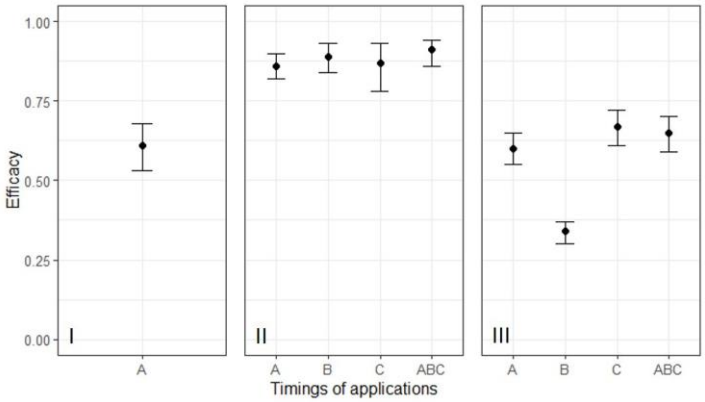

Figure 5.3. Efficacy of a fungicide applied at different timings in the growing season in reducing the sporulation potential of *Botrytis cinerea* on bunch trash (experiment 1). Bunch trash samples were collected 1 week after flowering in vineyard MA 2015 (**I**) and 1 week after veraison in vineyards CA in 2015 (**II**) and CO in 2016 (**III**). The fungicides was applied at A (full flowering; GS65 of Lorenz et al. 1995), B (pre-bunch closure; GS77), C (veraison; GS83), or ABC. The fungicide was a commercial mixture of fludioxonil (25%) and cyprodonil (37.5%) at 800 g/ha. Bars indicate the 95% confidence interval.

Colonization of bunch trash by *B. cinerea* as affected by product and timing of application

Weather conditions differed between the two years of experiment 2 (Fig. 5.2). The experimental period (i.e., between "inflorescences clearly visible" and "veraison"; GS53 and GS83, respectively) was 111 days long in 2017 and 87 in 2018 (Fig. 5.2). From GS53 to GS65, the average temperature was higher in 2018 than in 2017 (Fig. 5.2). In both years, the rain fell mostly between GS53 and GS77 (147.6 mm in 2017 and 204.4 in 2018), but the number of hours of wetness was higher in 2018 (608 h) than in 2017 (343 h; Fig. 5.2). In the last period, between GS77 and GS83, few rains were registered in either year (24.0 and 32.2 mm); however, the hours of wetness were consistently higher in 2018 (199 h of wetness) than 2017 (93 h of wetness) (Fig.5.2).

The average CC value for the NT bunch trash was 180 times higher in 2018 (9.22±3.72 CC) than in 2017 (0.05±0.01 CC), i.e., bunch trash colonization by *B. cinerea* was substantially higher in 2018 than in 2017 ($P<0.001$).

In 2017, the AIC of the three GLMs was lower for model 2 than for model 1 or 3 (Table 5.2). The selected model 2 showed no over-dispersion ($D/df=0.84$), and the assumptions of independence of errors, normality, and homoscedasticity of the residuals were confirmed (*not shown*). Model 2, in which both factors were considered (but not their interaction), indicated that the ranking of the products did not change over the different timings, but that their efficacy was influenced by timing. FUN significantly reduced bunch trash colonization compared to NT ($P=0.011$); BCA efficacy was not significantly different from that of FUN ($P=0.573$), while BOT efficacy was significantly lower than that of FUN or BCA ($P<0.001$) (Fig. 5.4l). The application at A had the same efficacy as applications at ABC ($P=0.468$), while efficacy was lower for applications at B and C than at ABC ($P=0.075$ and $P=0.057$, respectively) (Fig. 5.4l). Efficacy was highest with FUN and BCA applied at ABC, with confidence intervals of 0.60 to 0.95 and 0.46 to 0.94, respectively. Interestingly, the confidence intervals were shorter for FUN than for BCA, meaning that the variability among replicates was lower for FUN. The estimated efficacy for BOT applied at ABC was lower than for FUN and showed higher variability, ranging from 0.13 to 0.61 (Fig. 5.4l).

In 2018, models 4 and 5 had the lowest AIC values (Table 5.2); no over-dispersion was detected for either model ($D/df=0.58$ and 0.57, respectively), and assumptions of independence of errors, normality, and homoscedasticity of the residuals were confirmed (*not shown*). Model 5 was selected instead of model 4 to account for both product and timing. Unlike model 2 for 2017, model 5 for 2018 indicated that treatment efficacy was not influenced by product or timing. Specifically, FUN, BOT, and BCA all significantly reduced bunch trash colonization

by *B. cinerea* compared to NT (with P=0.065 for the null hypothesis that the efficacy of FUN is different from zero; P=0.197 and P=0.982 for BOT and BCA, respectively, for the null hypothesis that the efficacy of BOT or BCA is different from that of FUN), and were not significantly different from each other (P=0.358). Similarly, all of the timings of applications were similar to ABC (P=0.447 for A, P=0.509 for B, and P=0.712 for C). The overall efficacy values ranged from 0.25 to 0.96 (Fig. 5.4II).

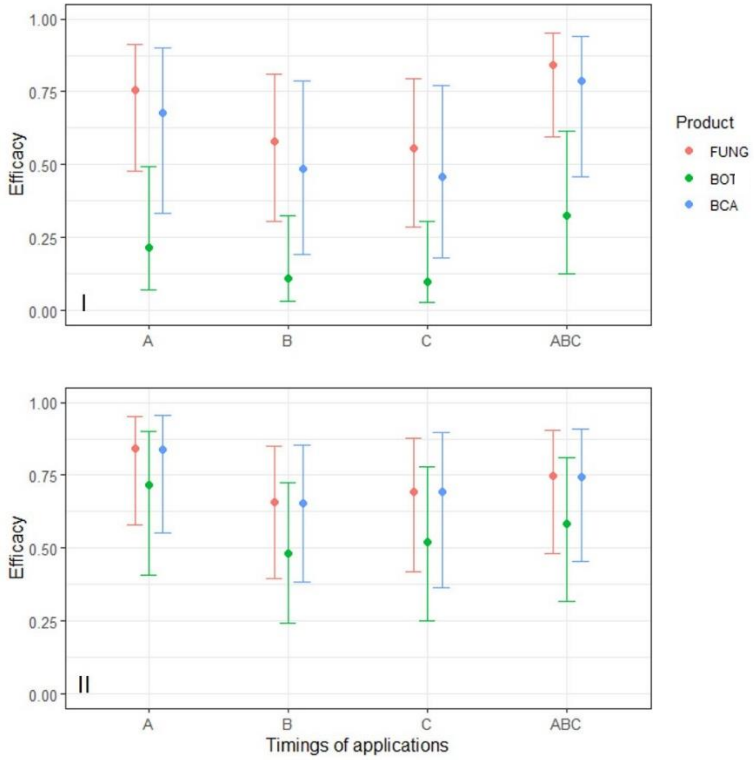

Figure 5.4. Efficacy of different products applied at different timings in reducing bunch trash colonization by *Botrytis cinerea* (experiment 2). Bunch trash samples were collected 1 week after veraison in vineyard CA in 2017 (**I**) and 2018 (**II**). Products were applied at A (full flowering; GS65 of Lorenz et al. 1995), B (pre-bunch closure; GS77), C (veraison; GS83), or ABC. The products were FUN, a commercial mixture of fludioxonil (25%) and cyprodonil (37.5%) at 800 g/ha; BOT, a commercial mixture of eugenol (3.2%), geraniol (6.4%), and thymol (6.4%) at 4000 ml/ha; and BCA, *A. pullulans* and *T. atroviride* at 400 g/ha and 1000 g/ha, respectively. Bars indicate the 95% confidence interval.

Discussion

Botrytis cinerea has a complex life cycle and attacks grapevines via multiple infection pathways; some of the pathways occur early in the season, i.e., from flowering to young cluster development (Elmer and Michailides 2007). One early season pathway involves the saprophytic colonization of bunch trash; this colonization has been traditionally considered a major source of inoculum within developing bunches (Nair and Hill 1992; Nair and Parker 1985), and correlations between bunch trash colonization by *B. cinerea* and BBR incidence at harvest have been reported (Seyb et al. 2000). As a consequence, *B. cinerea* chemical control in the early season, and especially at pre-bunch closure, has been recommended (Corvi and Tullio 1980; Pérez-Marín et al. 1998). However, the effects of these control interventions on bunch trash colonization by *B. cinerea* have seldom been studied for fungicides and have not been studied for biological agents or botanicals.

The current research used field experiments and laboratory assessments to determine how early season applications of fungicides (FUN), biological control agents (BCA), and botanicals (BOT) affect *B. cinerea* colonization of and sporulation on bunch trash. The results indicated that the ability of *B. cinerea* to colonize and sporulate on bunch trash was highly variable; this variability was higher between years than among vineyards and sampling times during the season. That the sporulation potential of *B. cinerea* on bunch trash changes over time has been previously observed (Balasubramaniam et al. 1998; Jaspers et al. 2012; 2016). This variability can be explained by the complex interactions between weather conditions and inoculum load, spore germination, and fungal growth on bunch trash (Ciliberti et al. 2015; 2016), and explaining the variability was not the objective of the current research. Instead, the current research used this variability to assess the effects of FUN, BCA, and BOT under very different conditions.

Concerning the effect of *B. cinerea* control on the bunch trash-related infection pathways, Calvo-Garrido et al. (2014b) quantified the incidence of *B. cinerea* colonization and sporulation potential in bunch trash at veraison to determine the effect of biological control agents applied at three times: 1-5% flowering, 80% flowering, and pre-bunch closure. Using laboratory incubations with optimal conditions for colonization and sporulation, the authors found that the biological control agents reduced the colonization of the bunch trash but not the sporulation potential on the bunch trash. Experiment 2 in the current study also revealed that application of BCA or FUN or BOT reduced the colonization of bunch trash by *B. cinerea*, based on the content of *B. cinerea* DNA in the trash and presented as a colonization coefficient (CC), in both years (which differed greatly in control CC values) and for all timings of applications (A, B, C, and ABC). This

effect was long-lasting because the bunch trash sprayed at A showed a reduction in CC 1 week after flowering and also after veraison. A previous study (Si Ammour et al. 2019, see Chapter 4) documented a positive relationship between CC and sporulation potential. Results from experiment 1 showed that FUN applications reduced the sporulation potential in only 3 of 18 cases, and that the probability of the applications being effective increased when the sporulation potential on the non-treated bunch trash increased, i.e., when the bunch trash colonization increased.

As was true for control of *B. cinerea* colonization of bunch trash, control of *B. cinerea* sporulation on bunch trash with an application at A was still effective 1 week after veraison, and this effect was greater than the application at B when the sporulation potential was high. Application at C also reduces *B. cinerea* colonization of bunch trash and the production of conidia during berry ripening and this confirms the important role of this application (González-Domínguez et al. 2019a, see Chapter 2). Application at A, i.e., early in the season, has previously been demonstrated to be important for reducing BBR severity at harvest in field experiments (Calvo-Garrido et al. 2014a); the importance of early season application was also documented in a meta-analysis of 116 studies (González-Domínguez et al. 2019a; 2019b, see Chapters 2 and 3). Fedele et al. (2018) showed that the efficacy of early season applications is related to a reduction in the incidence of latent infections (i.e., pathways I, IIa, and IIb of Elmer and Michailides 2007). The results of the current study indicate that the efficacy of application at A is also due to a reduction in bunch trash colonization and in subsequent sporulation (i.e., pathways III and IV; Elmer and Michailides 2007).

In this work, the effect of applications in reducing both the colonization and especially the sporulation potential of *B. cinerea* was highly variable. For the latter, the GLMs showed high overdispersion in the dataset, indicating that factors not accounted for by the experiment had an important effect on the results. These factors could include: i) the variability in *B. cinerea* colonization incidence among trash pieces in a bunch and among bunches; ii) the composition of bunch trash, because differences in the sporulation potential exist between bunch trash types, with tendrils and petioles supporting less sporulation than rachides (Jaspers et al. 2012); iii) the colonization severity (i.e., the amount of fungus) in the affected bunch trash pieces, which would be influenced by the inoculum load and weather conditions; and iv) the degree of depletion of nutritional resources in the bunch trash over time. All of these factors warrant further investigation and could account for that FUN applications significantly reduced *B. cinerea* sporulation in only 3 out of 18 cases. The results of experiment 2 showed that BCA had the same effect as FUN in reducing the colonization level and then the sporulation potential of bunch

trash, even though the efficacy was more variable with BCA than with FUN. The BCA application strategy used in this work was based on the mechanism of action (MoA) of the biological control agents: *Aureobasidium pullulans*, which was applied at A and C, is a good competitor for nutrients and can prevent germination of *B. cinerea* conidia; *Trichoderma atroviride*, which was applied at B, is a good colonizer of dead plant tissues and a competitor of *B. cinerea* for space and nutrients. This MoA-based application strategy controlled BBR at harvest in previous experiments with applications at B, C, and D (Pertot et al. 2017); the present work showed that this approach can be adopted for application at A. Results of experiment 2 showed that efficacy of BOT was inconsistent and showed variability in reducing bunch trash colonization. Even with repeated applications of the same BOT product as used in the current study, Rotolo et al. (2018) did not obtain satisfactory BBR control on table grapes at harvest. Nevertheless, botanicals are thought to have potential for controlling *B. cinerea* (Nguyen et al. 2013; Ribera et al. 2008), and further studies are needed to determine whether applications of BOT at A can reduce latent infections (i.e., pathway I, IIa, and IIb of Elmer and Michailides 2007).

Overall, spraying with FUN, BCA, or BOT at grape flowering may reduce the saprophytic colonization of bunch trash to different degrees and with some variability. For all products, colonization reduction was the same with application at A vs. ABC, meaning that the effect of an early season application lasted from flowering to 1 week after veraison. These results indicate that the early season control of *B. cinerea* is important to reduce the saprophytic colonization of bunch trash and the potential sporulation especially when the risk of BBR is high. So, an estimation of the risk of colonization during the early season would help growers decide whether an early spray application would reduce the sporulation potential later in the season. A mechanistic model that predicts the risk of *B. cinerea* development has recently been developed (González-Domínguez et al. 2015). This model, which is currently integrated in a Decision Support System (DSS) (Caffi et al. 2017; Rossi et al. 2012), predicts the relative infection risk during the two main grape-growing periods relevant for *B. cinerea* infection: (i) between ‘inflorescences clearly visible’ and ‘berries groat-sized, bunches begin to hang’; and (ii) ripening berries. The model is then able to assess the risk of bunch trash colonization in the early season. A qPCR assay for the quantification of colonization of bunch trash by *B. cinerea* may also be useful (Si Ammour et al. 2019, see Chapter 4). These tools (the model and the qPCR assay), combined with the findings of the current study, could improve BBR management in vineyards.

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Chapter 6

Chapter 6

Consideration of latent infections improves the prediction of Botrytis bunch rot severity in vineyards[1]

Abstract

The current study validated the mechanistic model for *Botrytis cinerea* on grapevine developed by González-Domínguez et al. (2015) with data from 23 independent Botrytis bunch rot (BBR) epidemics (combinations of vineyards × year) that occurred between 1997 and 2018 in Italy, France, and Spain. The model was operated for each vineyard by using weather data and vine growth stages to anticipate, at any day of the vine-growing season, the disease severity (DS) at harvest (severe, DS ≥15%; intermediate, 5< DS <15%; and mild, DS ≤5%). To determine the ability of the model to account for latent infections, post-harvest incubation assays were also conducted using mature berries without symptoms or signs of BBR. The model correctly classified the severity of 15 of 23 epidemics (65% of epidemics) when the classification was based on field assessments of BBR severity; when the model was operated to include BBR severity after incubation assays, its ability to correctly predict BBR severity increased from 65% to >87%. This result showed that the model correctly accounts for latent infections, which is important because latent infections can substantially increase disease severity. The model was sensitive and specific, with the false positive and false negative proportion of model predictions equal to 0.24 and 0, respectively. Therefore, the model may be considered a reliable tool for decision-making for BBR control in vineyards.

[1] Fedele G, González-Domínguez E, Delière L, Díez-Navajas AM, Rossi V, 2020. *Plant Disease*, DOI: 10.1094/PDIS-11-19-2309-RE

Introduction

Botrytis bunch rot (BBR), caused by the fungus *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel), is a serious disease in vineyards (Elmer and Michailides 2007; Williamson et al. 2007). The fungus affects all vine organs and especially clusters, and thereby reduces both the quantity and quality of the harvested grapes. Crop losses results from damage to inflorescences before flowering, to flowers during flowering, to young berries at fruit set, and to berries during ripening; the latter damage is referred to as bunch rot. Quality is reduced because rotted berries have an altered chemical composition that causes undesirable flavors in wine (Steel et al. 2013).

Botrytis cinerea develops and actively grows as a necrotrophic pathogen and as a saprophyte on different substrates (Elmer and Michailides 2007). The pathogen overwinters on herb debris, bunch and leaf trash, and rotted berries, and large numbers of conidia can be produced on these overwintering sites under a wide range of environmental conditions (Ciliberti et al. 2015a; 2016). The multiple infection pathways occur in two periods: between flowering to young cluster development, and after veraison (Elmer and Michailides 2007). During the first period, conidia infect inflorescences and young berries through three pathways: in pathway I, conidia infect the styles and ovules; in pathway IIa, conidia infect the stamens or petals; and in pathway IIb, conidia infect fruit via fruit pedicels (Elmer and Michailides 2007). These infections can cause either blossom blight or latent infections of berries. During the flowering stage, in pathway III, conidia infect and saprophytically colonize bunch trash (the dead stamens, aborted flowers, aborted berries, calyptras, and tendrils) retained within the developing bunches (Elmer and Michailides 2007). In the early season, infection severity increases with hours of wetness at temperatures near 20°C (Ciliberti et al. 2015a; 2015b; 2016; Latorre and Rioja 2002; Nair and Allen 1993). During the second period, latent infections may become visible as rotted berries and may contribute to final disease severity, but the contribution of latent infections to final disease remains unclear (Keller et al. 2003; McClellan and Hewitt 1973). In addition, the mycelium colonizing the bunch trash can produce conidia under favorable conditions, and in pathway IV, the conidial accumulation within the developing bunch results in a source of inoculum for new infections of the ripening berries (Elmer and Michailides 2007). In pathway Va, wind-dispersed conidia infect ripening berries (Elmer and Michailides 2007) and in pathway Vb, the aerial mycelium produced on adjacent infected berries infects ripening berries through berry-to-berry contact (González-Domínguez et al. 2015). From veraison to ripening, the risk of infection is highest at temperatures between 15 and 25°C and also increases with hours of wetness or high relative humidity (Broome et al. 1995; Ciliberti et al. 2015b; Latorre and

Rioja 2002; Nair et al. 1988; Nair and Allen 1993). The appearance of symptoms is also promoted by the increasing susceptibility of berries approaching maturity (Deytieux-Belleau et al. 2009; Hills et al. 1981; Kretschmer et al. 2007; Mundy and Beresford 2007).

In spite of the complexity of the life cycle of the pathogen, the management of the disease is commonly based on a routine calendar application of fungicides at four specific grape growth stages (GS): A, end of flowering (GS69; Lorenz et al. 1995); B, pre-bunch closure (GS77); C, veraison (GS83); and D, before harvest (before GS89). This schedule may result in unnecessary sprays because the applications are preventive and do not take into account the real risk of BBR infections (González-Domínguez et al. 2019a, see Chapter 2). To predict the disease risk and help growers in deciding whether a fungicide application is needed, González-Domínguez et al. (2015) developed a mechanistic model according to the principles of "systems analysis" (Leffelaar and Ferrari 1989). The model was previously evaluated with data collected from 21 vineyards in Italy and France and between 2009 and 2014; according to a discriminant function analysis (DFA), the model correctly classified 81% of the epidemics (González-Domínguez et al. 2015).

The validation conducted by González-Domínguez et al. (2015), however, used the same dataset that was used to develop the model. It follows that additional validation is needed using an independent dataset (Rossi et al. 2010). Moreover, the model developed by González-Domínguez et al. (2015) indirectly estimates latent infections considering the risk of infections in the early-season but the previous validation was conducted by using only BBR severities at cluster maturity in vineyard and, the ability of the model to account for those infections that are still latent at harvest was not assessed. In the present study, the model was further validated with data from 23 independent BBR epidemics that occurred between 1997 and 2018 in Italy, France, and Spain. Latent infections of mature berries were also assessed to determine the ability of the model to account for latent infections

Materials and methods

Vineyards

Data were collected between 1997 and 2018 from plots not treated for BBR in eight experimental vineyards: three in Italy, three in France, and two in Spain. A total of 23 epidemics (combinations of vineyard × year) were considered (Table 6.1). Vineyards were cropped with grape varieties susceptible to *B. cinerea* (Table 6.1) and were managed as usual for the viticultural area, with the exception that no fungicides were used to control *B. cinerea*. Weather data were collected using

standard electronic weather stations placed along the vineyard borders (with sensors at 1.5 m above the ground). Temperature (ºC), relative humidity (%), wetness duration (hours), and rainfall (mm) were measured hourly.

Field assessments

Growth stages (GS) of vines were assessed twice each week, from inflorescences clearly visible (GS53) until harvest (GS>89) according to Lorenz et al. (1995). At full ripening (GS89), BBR incidence (DI) and severity (DS) were visually assessed on a minimum of 100 random bunches per plot (on at least 20 vines per plot), in at least four replicate plots per vineyard. DI was calculated as the percentage of bunches with BBR, and DS was calculated as the percentage of the bunch surface affected by BBR (Hill et al. 2010).

Incubation assays

In 10 of the 23 epidemics that did not show any BBR symptoms in the field (Table 6.2), incubation assays were conducted to assess the presence of latent infections in ripening berries. In each epidemic, 100 berries (with their pedicel) that did not show any symptoms or sign of rot were randomly collected just before harvest (GS89). Berries were transported to the laboratory in a cooler, and were rinsed under tap water for 3 min, disinfested with 2/3 of distilled water and 1/3 of 5% sodium hypochlorite to remove epiphytic microflora, and finally rinsed again with sterile water. Berries were placed in a metallic box (20 × 15 cm, with a wet filter paper on the bottom) over a metallic grid net so that berries did not touch each other or the filter paper. Each of four replicate boxes contained 25 berries. The boxes were sealed in plastic bags to maintain a saturated atmosphere and were incubated at 25°C with a 12-h photoperiod for 7 days. DI was then assessed as the percentage of berries showing typical rotting and *B. cinerea* sporulation; the percentage of the surface of each berry affected by BBR was also evaluated. The average severity was then calculated as sum of disease severity in single (affected) berries divided by the total number of berries (healthy + affected), and a value of DS after incubation assays was tassigned based on the standard area diagram of Hill et al. (2010).

Model structure and running

The model was described by González-Domínguez et al. (2015). In brief, the model begins to operate when grape inflorescences are clearly visible (GS53) and ends at harvest (GS90), with a time step of 1 day. The model assumes that inoculum sources are present in the vineyard and estimates the relative abundance of conidia produced on inoculum sources on any day of the grape-

growing season as a function of the rate at which the mycelium grows and saprophytically colonizes the source tissue and produces conidia. The model then assumes that, on any day, mature conidia may disperse and settle on host plant surfaces. The model considers two main infection periods. In the first infection period (between GS53 “inflorescences clearly visible” and GS73 “berries groat-sized, bunches begin to hang”), the model calculates a daily infection risk (RIS1) for infections by conidia on inflorescences and young clusters (pathways I and II of Elmer and Michailides 2007). In the second infection period (between GS79 “majority of berries touching” and GS89 “berries are ripe for harvest”), the model calculates the daily infection risk on ripening clusters for infections caused by conidia (pathway Va of Elmer and Michailides 2007; RIS2) and for berry-to-berry infection by aerial mycelium (pathway Vb of González-Domínguez et al. 2015; RIS3). Daily values of infection risk are accumulated over the time of the corresponding infection period, and accumulated values produce new variables (SEV1, SEV2, and SEV3), which contribute to the total risk of infection. The model finally uses SEV1, SEV2, and SEV3 as independent variables in a discriminant function analysis (DFA) to classify the epidemics in three groups based on disease severity (DS) at harvest: severe, (DS ≥15%), intermediate (5< DS <15%), or mild (DS ≤5%).

For each vineyard, the model was operated using the vineyard’s weather data and vine growth stages to predict, on any day of the vine-growing season, the epidemic group (severe, intermediate, or mild) at harvest (González-Domínguez et al. 2015).

Data analysis

Observed BBR epidemics were classified into three groups based on disease severity (DS) at harvest in field and after incubation assays as follows: severe, DS ≥15%; intermediate, 5≤ DS <15%; mild, DS <5% (Table 6.2). These observed BBR severities (O) were compared with those predicted by the model (P). A 2×2 contingency table was then built, in which P and O were categorized as either DS <5% or DS ≥5%, by using three different datasets: i) the 23 epidemics classified based on the DS in field and predicted by the model (*dataset 1*); ii) the 10 epidemics classified based on the DS after incubation assays and predicted by the model (*dataset 2*); and iii) the 23 epidemics classified based on the DS after incubation assays or in field and predicted by the model (*dataset 3*) (Table 6.2).

Predictions were classified as follows: i) true positive, when the epidemics predicted by the model and observed (in the field or in the incubation assay) were both classified as intermediate or severe, i.e., DS ≥5% (P+,O+); ii) true negative, when the predicted and observed epidemics were both mild, i.e., DS <5% (P−,O−);

iii) false positive, when the predicted epidemics were intermediate or severe, but the observed epidemics were mild (P+,O−); and iv) false negative, when the predicted epidemics were mild, but the observed epidemics were intermediate or severe (P−,O+). The true positive proportion (TPP or sensitivity), the true negative proportion (TNP or specificity), the false positive proportion (FPP), and the false negative proportion (FNP) were then calculated (Madden 2006). The overall accuracy of the predictions was calculated as the ratio between correct and total predictions. Bayesian analyses were run to calculate the following posterior probabilities (Yuen and Hughes 2002; Madden 2006): (i) the probability that the observed epidemic was intermediate or severe when predicted to be intermediate or severe, P(P+|O+); (ii) the probability that the observed epidemic was mild when predicted to be mild, P(P−|O−); (iii) the probability that the observed epidemic was mild when predicted to be intermediate or severe (i.e., the model provided unjustified alarms), P(P+|O−); and (iv) the probability that the observed epidemic was intermediate or severe when predicted to be mild (i.e., the model does not predict real infections), P(P−|O+). These posterior probabilities were compared with the prior probabilities. Prior probabilities were calculated as the proportion of intermediate or severe epidemics P(O+) or mild epidemics P(O−) relative to the total number of epidemics observed in field.

Results

BBR severity observed in the field at maturity ranged from DS=0% to 52.4% (Table 6.2). Of the 23 BBR epidemics, 16 were mild (with average DS=1.1±0.5), 1 was intermediate (with DS=11.6), and 6 were severe (with average DS=39.7±3. 6) (Table 6.2). During the first infection period (i.e., from GS53 “inflorescences clearly visible” to GS73 “berries groat-sized, bunches begin to hang”), the average temperature ranged from 15.8 to 19.9ºC (Table 6.1), rain ranged from 22.4 to 275.5 mm, and wetness duration ranged from 58 to 614 (Table 6.1). In the second infection period (i.e., from GS79 “majority of berries touching” to GS89 “berries are ripe for harvest”), average temperatures were higher (from 18.2 to 26.2°C) than in the first infection period, rain ranged from 35.8 and 223.6 mm, with wetness duration ranged from 35 to 940 h (Table 6.1).

The daily model outputs for the 23 BBR epidemics are presented in Figure 6.1 in terms of severity categories (mild, intermediate, or severe). Eight of these epidemics (CA-16, CA-15, CA-17, MA-17, LG-16, PA-10, LA-03, and PA-11) were predicted to be mild all season long (Fig. 6.1). The other epidemics were predicted to be intermediate starting from the second week of May (CA-18, CO-16, CO-17, MA-16, MA-15, PA-07, PA-15, LA-02, PA-08, and PA-14) to mid-June (ZA-17 and PA-13), which was between 27 and 2 days before GS69 (i.e., end of flowering; Fig.

6.1). Seven epidemics were then predicted to be severe (CA-18, PA-07, ZA-16, PA-15, LA-02, and PA-13, PA-14) after GS83 (i.e., veraison), between 15 and 44 days before GS89 (i.e., berries ripe for harvest) (Fig. 6.1). In epidemics CO-16 and ZA-17, the change from intermediate to severe occurred at GS69, between 112 and 96 days before GS89; in CO-15 and LS-97, the classification jumped from mild to severe (Fig. 6.1).

When the classification of BBR epidemics based on field assessments and model outputs were compared (*dataset 1*), 15 of the 23 epidemics were correctly classified, so that overall accuracy was 0.65 (Table 6.3). In the 8 misclassifications, observed epidemics were mild, but the model classified them as intermediate (3 cases) or severe (5 cases) (Table 6.2), so that FPP was 0.50 (Table 6.3). The model correctly classified mild or severe epidemics in 8 of 16 cases, so that TNP was 0.50, and all of the observed epidemics were mild, so that TPP was 1 and FPP was 0 (Table 6.3). Based on the Bayesian analysis, the posterior probability that the model correctly predicted an intermediate or severe epidemic, P(P+|O+), was 0.47; the posterior probability that the model correctly predicted a mild epidemic, P(P−|O−), was 1.00 (Table 6.3).

For the 10 epidemics with no BBR at harvest but which included incubation assays, BBR severity without consideration of incubation assays was nil in the field (as indicated earlier) and was therefore classified as mild; based on the model, these epidemics were predicted to be mild in 4 cases and intermediate or severe in 6 cases (Table 6.2), so that overall accuracy was 0.40. If data from the incubation assays were included (*dataset* 2), DS ranged 1 to 15%, and the epidemics were classified as mild in 5 cases and intermediate or severe in 5 cases (Table 6.2), so that 9 of these epidemics were correctly classified by the model, and the overall accuracy of the model increased to 0.90 (Table 6.3). The only misclassified epidemic was predicted to be severe but was observed to be mild (DS=1.1%), i.e., the FPP = 0.20 (Table 6.3). In the Bayesian analysis of *dataset 2*, the posterior probability of correctly predicting mild epidemics, P(P−|O−), was 1.00, and of correctly predicting intermediate or severe epidemic, P(P+|O+), was 0.83.

When all 23 observed epidemics were classified based on DS (these classifications included incubation assays for 10 epidemics and field assessment at harvest for 13 epidemics) and compared with model output (*dataset* 3), 20 of 23 epidemics were correctly classified, i.e., overall accuracy was 0.87 (Table 6.3). The three epidemics that were incorrectly classified were mild in the field (with DS=0%) but were classified as severe by the model; two of these epidemics (PA-07 and ZA-16) were only assessed in the field, and only one included an incubation assay (CA-18) (Table 6.2). The posterior probability of correctly predicting intermediate or severe epidemics, P(P+|O+), was 0.80, and was higher for *dataset*

3 than for *dataset 1* (which only included field assessment) (P(P+|O+)=0.47), while the posterior probability of correctly classifying mild epidemics remained P(P+|O−)=1.00 (Table 6.3), indicating that the consideration of latent infections in the assessment of BBR severity greatly improved the predictive ability of the model.

Table 6.1. Characteristics of the vineyards and summary of the weather data recorded in the first and second infection periods of *Botrytis cinerea* considered by the model of González-Domínguez et al. (2015) for Botrytis bunch rot (BBR).

				1st infection period[b]			2nd infection period[c]		
Epidemic	**Location (country)[a]**	**Year**	**Cultivar**	**Temperature (°C)[d]**	**Rain (mm)[e]**	**Wetness duration (h)[f]**	**Temperature (°C)**	**Rain (mm)**	**Wetness duration (h)**
LS-97	La Sauve (FR)	1997	Sauvignon	17.2	147.0	334	22.4	130.5	415
LA-02	Latresne (FR)	2002	Sauvignon	17.2	135.2	535	19.4	139.8	343
LA-03	Latresne (FR)	2003	Sauvignon	17.8	118.6	363	24.7	65.8	175
PA-07	Pauillac (FR)	2007	Merlot	17.1	163.4	393	19.8	128.6	347
PA-08	Pauillac (FR)	2008	Merlot	16.9	222.2	376	20.1	152.6	374
PA-10	Pauillac (FR)	2010	Merlot	16.2	251.4	336	19.6	152.8	273
PA-11	Pauillac (FR)	2011	Merlot	17.4	22.4	92	20.2	154.7	199
PA-13	Pauillac (FR)	2013	Merlot	15.8	162.5	532	18.6	101.5	663
PA-14	Pauillac (FR)	2014	Merlot	15.9	159.7	540	20.0	72.5	625
CA-15	Castell'Arquato (IT)	2015	Merlot	18.8	103.4	206	26.2	66.0	85
CO-15	Cormons (IT)	2015	Merlot	18.9	178.2	255	25.5	223.6	213
MA-15	Mandriole (IT)	2015	Trebbiano Romagnolo	19.9	109.7	338	24.7	50.9	295
PA-15	Pauillac (FR)	2015	Merlot	16.7	114.4	461	20.3	195.8	450
CA-16	Castell'Arquato (IT)	2016	Merlot	16.8	273.0	178	24.4	97.4	60
CO-16	Cormons (IT)	2016	Merlot	16.5	275.5	614	24.2	108.2	181
MA-16	Mandriole (IT)	2016	Trebbiano Romagnolo	17.1	184.2	446	23.5	53.3	475
LG-16	La guardia (SP)	2016	Tempranillo	17.7	30.4	58	18.9	35.8	35
ZA-16	Zalla (SP)	2016	Hondarrabi Zuri	16.2	105.0	392	19.1	158.4	791
CA-17	Castell'Arquato (IT)	2017	Merlot	17.4	120.2	300	25.8	43.2	87
CO-17	Cormons (IT)	2017	Merlot	16.9	155.5	430	25.0	108.8	410
MA-17	Mandriole (IT)	2017	Trebbiano Romagnolo	17.9	81.4	115	24.1	70.0	170
ZA-17	Zalla (SP)	2017	Hondarrabi Zuri	15.4	192.6	813	18.2	177.8	940
CA-18	Castell'Arquato (IT)	2018	Merlot	18.7	153.8	485	24.9	77.8	412

[a] Country code: IT= Italy, FR= France, SP= Spain.
[b] The first infection period extends from GS53 "inflorescences clearly visible" to GS73 "berries groat-sized, bunches begin to hang".
[c] The second infection period extends from GS79 "majority of berries touching" to GS89 "berries are ripe for harvest".
[d] Average of daily temperatures (°C).
[e] Total mm of rain (mm).

Table 6.2. Incidence and severity of Botrytis bunch rot (BBR) in the field and after the incubation assay, and the classifications of the observed and the predicted epidemics for three datasets[a].

Epidemic	Observed							Predicted
	Field			Incubation assay			Group[d] *Dataset 3*	Group[e]
	DI[b] (%)	DS[c] (%)	Group[d] *Dataset 1*	DI[b] (%)	DS[c] (%)	Group[d] *Dataset 2*		
LS-97	82.9	36.8	S				S	S
LA-02	87.3	37.1	S				S	S
LA-03	30.8	4.7	M				M	M
PA-07	16.2	1.4	M				M	S
PA-08	96.1	45.9	S				S	S
PA-10	46.9	2.6	M				M	M
PA-11	30.4	4.9	M				M	M
PA-13	90.3	39.2	S				S	S
PA-14	94.8	52.4	S				S	S
CA-15	0.0	0.0	M	35.5	3.0	M	M	M
CO-15	0.0	0.0	M	77.5	9.0	I	I	S
MA-15	0.0	0.0	M	50.0	10.0	I	I	I
PA-15	81.6	26.8	S				S	S
CA-16	0.0	0.0	M	14.0	3.0	M	M	M
CO-16	0.0	0.0	M	56.0	15.0	S	S	S
MA-16	0.0	0.0	M	7.0	5.0	I	I	I
LG-16	2.4	0.1	M				M	M
ZA-16	55.6	4.1	M				M	S
CA-17	0.0	0.0	M	14.0	1.0	M	M	M
CO-17	0.0	0.0	M	63.0	9.0	I	I	I
MA-17	0.0	0.0	M	36.0	2.0	M	M	M
ZA-17	82.5	11.6	I				I	S
CA-18	0.0	0.0	M	59.0	1.1	M	M	S

[a] Dataset 1 considers the 23 epidemics that were classified based on DS assessed only in the field, i.e., without data on disease that developed in the incubation assay; dataset 2 considers the 10 epidemics that were classified based on the DS assessed in the incubation assay (these epidemics has no bunch rot in the field); and dataset 3 considers all 23 epidemics, which were classified based on the DS assessed only in the field (for 13 epidemics) or in the field and also in the incubation assay (for 10 epidemics).

[b] Disease incidence assessed as the percentage of bunches or berries affected by Botrytis bunch rot in the field and in the incubation assay, respectively; the incubation assay was conducted only for 10 epidemics.

[c] Disease severity assessed as the percentage of the bunch surface affected by Botrytis bunch rot in the field or in the incubation assay.

[d] Epidemic groups are: S=severe (DS ≥15%), I=intermediate (5%≤ DS <15%), M=mild (DS <5%).

[e] Epidemic group predicted by the DFA described in González-Domínguez et al. (2015).

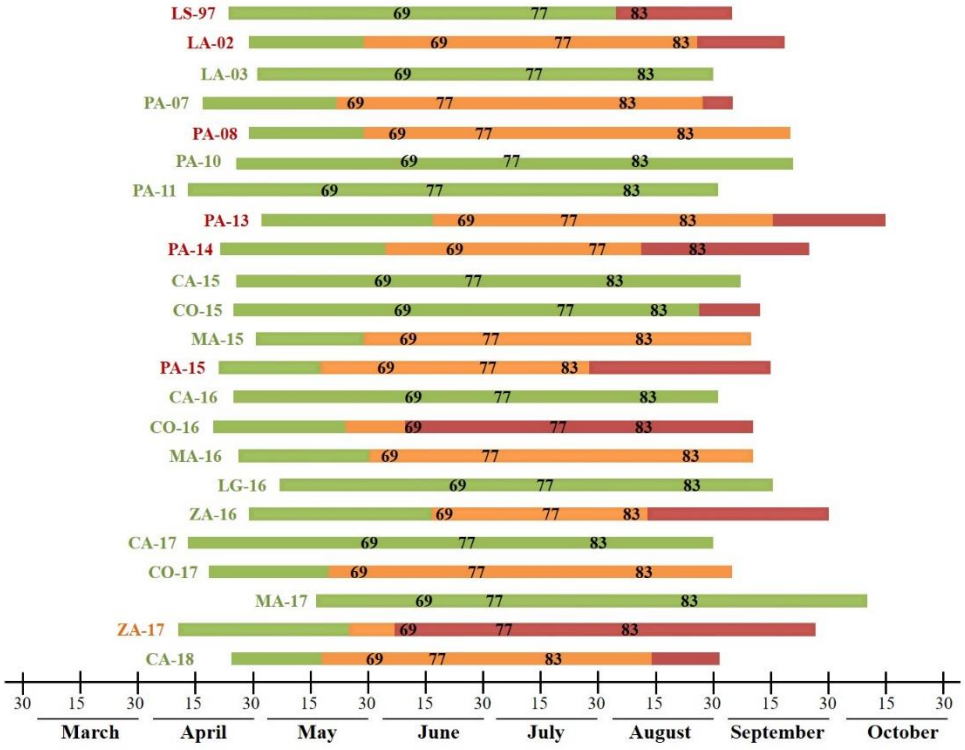

Figure 6.1. Classification of 23 BBR epidemics of Table 2 as mild (green horizontal bars), intermediate (orange bars), or severe (red bars) based on the model of González-Domínguez et al. (2015), which was operated daily "inflorescences clearly visible" (GS53 of Lorenz et al. 1995) and "berries ripe for harvest" (GS89). The code of the epidemics (e.g., LS-97) on the left of each horizontal bar is also colored based on the observed BBR severity assessed in the vineyard at harvest, being green, orange, or red when DS <5%, 5≤ DS <15%, and DS ≥15%, respectively. Numbers inside bars indicate the critical growth stages for fungicide applications: 69 = end of flowering; 77 = pre-bunch closure; and 83 = veraison.

Table 6.3. Bayesian statistics of the classification of the 23 Botrytis bunch rot epidemics of Table 6.1 as mild or intermediate/severe based on field assessments of disease severity in the field and/or in the incubation assay (observed) and on the model developed by González-Domínguez et al. (2015) (predicted).

Dataset[a]	Observed[b]	Predicted[c]		Accuracy[d]	Likelihood ratio (LR)		Prior probability (P)[e]		Posterior probability (P)[f]			
		Yes (P+)	No (P–)									
1	**Yes (O+)**	7 (1.00)[g]	0 (0.00)[h]	0.65	**LR(O+)**	2.00	**P(O+)**	0.30	**P(P+|O+)**	0.47	**P(P–|O+)**	0.00
	No (O–)	8 (0.50)[i]	8 (0.50)[l]		**LR(O–)**	0.00	**P(O–)**	0.70	**P(P+|O–)**	0.53	**P(P–|O–)**	1.00
2	**Yes (O+)**	5 (1.00)	0 (0.00)	0.90	**LR(O+)**	5.00	**P(O+)**	0.50	**P(P+|O+)**	0.83	**P(P–|O+)**	0.00
	No (O–)	1 (0.20)	4 (0.80)		**LR(O–)**	0.00	**P(O–)**	0.50	**P(P+|O–)**	0.17	**P(P–|O–)**	1.00
3	**Yes (O+)**	12 (1.00)	0 (0.00)	0.87	**LR(O+)**	3.67	**P(O+)**	0.52	**P(P+|O+)**	0.80	**P(P–|O+)**	0.00
	No (O–)	3 (0.27)	8 (0.73)		**LR(O–)**	0.00	**P(O–)**	0.48	**P(P+|O–)**	0.20	**P(P–|O–)**	1.00

[a] Dataset 1 considers the 23 epidemics that were classified based on DS assessed only in the field, i.e., without data on disease that developed in the incubation assay; dataset 2 considers the 10 epidemics that were classified based on the DS assessed in the incubation assay (these epidemics has no bunch rot in the field); and dataset 3 considers all 23 epidemics, which were classified based on the DS assessed only in the field (for 13 epidemics) or in the field and also in the incubation assay (for 10 epidemics).

[b] Observed membership is based on disease severity of BBR assessed in the field at maturity. "No" are mild epidemics with disease severity (DS) <5%; "Yes" are intermediate or severe epidemics with disease severity (DS) ≥5%.

[c] Membership predicted by the model developed by González-Domínguez et al. (2015). "No" are mild epidemics; "Yes" are intermediate or severe epidemics.

[d] Proportion of correct predictions calculated as (P+,O+/P–,O–)/total cases.

[e] Prior probabilities for intermediate or severe (O+) and mild (O−) epidemics.

[f] Posterior probabilities: (i) the probability that the observed epidemic was intermediate or severe when predicted to be intermediate or severe, P(P+|O+); (ii) the probability that the observed epidemic was mild when predicted to be mild, P(P−|O−); (iii) the probability that the observed epidemic was mild when predicted to be intermediate or severe (i.e., the model provides unjustified alarms), P(P+|O−); (iv) the probability that the observed epidemic was intermediate or severe when predicted to be mild (i.e., the model does not predict real infections), P(P−|O+).

[g] Number of cases; in brackets the true positive proportion (TPP, or sensitivity), when the predicted and observed epidemics were both classified as intermediate or severe (P+,O+).

[h] Number of cases; in brackets the false negative proportion (FNP), when the predicted epidemics were mild, but the observed epidemics were intermediate or severe (P–,O+).

[i] Number of cases; in brackets the false positive proportion (FPP), when the predicted epidemics were intermediate or severe, but the observed epidemics were mild (P+,O–).

[l] Number of cases; in brackets the true negative proportion (TNP, or specificity), when both the predicted and observed epidemics were mild (P–,O–).

Discussion

As noted in the Introduction, González-Domínguez et al. (2015) developed a mechanistic model that predicts *B. cinerea* development in grapevines and predicts the severity of BBR at harvest as mild (DS <5%), intermediate (5≤ DS <15%), or severe (DS ≥15%). As part of the same study, González-Domínguez et al. (2015) cross-validated the model using a dataset composed of 21 epidemics. Although the cross-validation indicated an overall model accuracy of >80%, the cross-validation used the same data set that was used to develop the model. In the current study, the model was further validated by using an independent dataset based on 23 epidemics (combinations of vineyard × year) in Italy, France, and Spain. The model's ability to predict BBR epidemics as mild, intermediate, or severe was lower when based on the independent dataset rather than on the dataset used by González-Domínguez et al. (2015), i.e., only 65% of the epidemics were correctly classified. Specifically, the model (without consideration of latent infections as determined by incubation assays) misclassified 50% of the mild epidemics (i.e., the epidemics with DS <5%); most of these observed epidemics lacked BBR symptoms at harvest but were predicted to be intermediate or severe by the model. This misclassification refers to false positive predictions (Madden 2006), which may result in unjustified alarms and therefore in unjustified fungicide sprays (Shtienberg 2007). Unjustified sprays should be avoided in order to reduce fungicide costs and fungicide effects on the environment (Epstein 2014) and public health (Alavanja et al. 2004; Verger and Boobis 2013), and in order to reduce the risk that resistance to fungicides develops in *B. cinerea* populations (Fernández-Ortuño et al. 2016; Fillinger and Walker 2016; Leroux 2007).

When the model considered BBR severity based both on data obtained at harvest and after incubation assays, its ability to correctly predict the epidemics as mild, intermediate, or severe increased from 65% to >87%. In our incubation assays, grape berries without visible symptoms or signs of *B. cinerea* at harvest were kept at a favorable temperature and humidity so that latent infections could become visible; in other words, the consideration of BBR severity after incubation assays for model validation meant the consideration of those latent infections that did not result in BBR symptoms at harvest under field conditions. That latent infections are important in *B. cinerea* epidemiology has been clearly demonstrated (Keller et al. 2003; McClellan and Hewitt 1973; Nair et al. 1995; Pezet and Pont 1984). McClellan and Hewitt (1973) and Pezet and Pont (1984) were the first reporting that berries develop latent infections as early as at flowering, and that these infections remain latent (not visible) until berries ripen, and in some cases are still latent at harvest. Keller et al. (2003) and Nair et al. (1995) found that the majority of rotted berries at harvest had become infected during flowering. The

conditions that cause latent infections to result in the visible rotting of berries are not completely understood; several factors may be important, such as meteorological conditions and especially intense rainfall, vineyard and cultivar characteristics, or the presence of wounds or cracks on the berry skin (Elmer and Michailides 2007; Mundy and Beresford 2007; Nair et al. 1988; Nelson 1951; Jarvis 1977).

The improvement of the overall accuracy of the model-based prediction of BBR epidemics from 65% (based on field data only) to >87% (based on field data and incubation assays) showed that the model is able to account for latent infections and to therefore correctly represent the complexity of *B. cinerea* epidemiology in vineyards. Specifically, the model correctly predicted 20 of 23 epidemics, with three epidemics being classified as mild based on observed data but as severe based on the model. In two of these epidemics (PA-07 and ZA-16), DS was assessed in the field only (as 1.4 and 4.1%, respectively), and it is possible that incubation assays, had they been conducted, may have increased the total BBR severity to >5% (which would account for the severity in field plus the severity after incubation assays). Therefore, the false positive proportion of model predictions was very low, and the false negative proportion was nil. Based on that, the model can be considered sensitive and specific (Madden 2006), and may be considered a reliable tool for supporting decision making for BBR control in vineyards.

One might argue that a model is not very useful if it advises the need for disease control based on the risk of latent infections even though these infections may remain latent through ripening and harvest (as was the case in 10 epidemics in this research). The control of latent infections, however, is always useful for three reasons. First, latent infections that establish between flowering and fruit set represent an important source of inoculum inside the cluster during ripening, when they initiate the rotting of berries (pathway I, IIa, and IIb of Elmer and Michailides 2007) (Calvo-Garrido et al. 2014); because the reason(s) why latent infections initiate the rotting of berries during ripening in some cases but not in others are unknown, controlling latent infections is warranted. Second, the advantages of early-season control of *B. cinerea* have been clearly demonstrated (González-Domínguez et al. 2019a; 2019b, see Chapters 2 and 3) and affect other infection pathways such as pathways III (saprophytic colonization of bunch trash) and IV (spore production on bunch trash) of Elmer and Michailides (2007) (Calvo-Garrido et al. 2014; Fedele et al. 2019, see Chapter 5), in addition to the latent infection pathways I, IIa, and IIb (Elmer and Michailides 2007). Third, latent infections can alter the chemical properties of berry juices (Steel et al. 2013); in particular, they can increase the contents of glycerol and gluconic acid, which are used as

indicators of the negative effects of BBR on grape juice and wines (Nigro and Versari 2008).

In conclusion, the current study validated a mechanistic BBR model recently developed by González-Domínguez et al. (2015). Use of this model could improve BBR management in vineyards by helping farmers schedule fungicides based on the predicted risk of disease. During the season, the model could advise farmers as to whether the current weather conditions are favorable for *B. cinerea* infection and will lead to a final BBR severity >5%, which is considered a threshold for bunch damage (González-Domínguez et al. 2015). By using the model, farmers would apply fungicides only when the predicted final BBR severity exceeds the threshold; this would prevent the needless application of fungicides. The model is currently available for growers in vite.net, which is a decision support system for the sustainable management of vineyards (Caffi et al. 2017; Rossi et al. 2012).

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Chapter 7

Chapter 7

Influence of environment on the biocontrol of *Botrytis cinerea*: a systematic literature review [1]

Abstract

The biocontrol of grey mould, caused by the fungus *Botrytis cinerea,* has been intensively studied in the last decades, and biological control agents (BCAs) have been developed as active ingredients of several currently available products. However, the biocontrol of grey mould remains challenging, and farmer reliance on BCAs remains marginal. One likely reason is the inconsistent efficacy of BCAs under field conditions, which may be related to several factors, including the variability of the environmental conditions affecting both BCA fitness and *B. cinerea* development. A systematic literature review was conducted to retrieve and analyze the metadata on the influence of environmental conditions on BCA fitness and efficacy against *B. cinerea*. The review considered 54 papers (selected from a total of 347 papers) and 27 genera of BCAs. The review showed that only limited information is available about the effects of temperature, humidity, and pH on BCA fitness and efficacy. Metadata were used to define environmental niches for *B. cinerea* and for two BCAs, *Trichoderma* and *Candida*, which were used as case studies. The environmental niches, in turn, were used to study the temperature and humidity conditions under which the BCA prevails over *B. cinerea*, and to define the extent of environmental niche sharing between the BCA and the target pathogen. Possible uses of environmental niches for improving BCA efficacy are discussed.

[1] Fedele G, González-Domínguez E, Rossi V, 2019. Submitted as book chapter to Springer Netherlands ed. by A De Cal, P Melgarejo, N Magan, In Strategies to develop successful Biocontrol Agents;.

Introduction

Grey mould, caused by *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel), is a serious disease of many economically important crops including fruits, vegetables, and flowers. This pathogen causes considerable yield and quality losses in field production and storage worldwide (Jarvis 1977; Williamson et al. 2007). Control of the disease is difficult because *B. cinerea* has high genetic variability, a short life cycle, a high reproductive rate (Elad et al. 2007), and multiple infection pathways (Elmer and Michailides 2007). The pathogen is able to survive and sporulate as a saprophyte on necrotic tissue (Elad et al. 2007). For these reasons, farmers strongly rely on fungicides for the control of *B. cinerea*. Strict dependence on fungicides, however, is not sustainable for two main reasons. First, the public is increasingly concerned about the effects of chemicals on human health and the environment (Alavanja et al. 2004; Epstein 2014). Second, *B. cinerea* populations frequently develop resistance to fungicides (Leroux 2007), which is difficult to avoid with the current resistance-management strategies; these strategies are based mainly on the alternating or mixing of fungicides with different modes of action (Fernández-Ortuño et al. 2015; 2016).

In recent years, researchers have been increasingly exploring alternatives to chemical control, including the use of microorganisms like yeasts, fungi, and bacteria that may suppress *B. cinerea* via competition, antibiosis, and/or parasitism (Elmer and Reglinski 2006; Elad and Stewart 2007; Haidar et al. 2016). These microorganisms are termed biological control agents (BCAs) and have the potential to complement and replace chemicals both before and after crop harvest. The substantial interest in BCAs against *B. cinerea* is demonstrated by the more than 2000 relevant papers listed in the Web of Sciences[1].

Most of these papers focused on the evaluation of the *in vitro* interaction between the BCA and *B. cinerea* in artificial media and under specific, usually constant environmental conditions (e.g., constant temperature regimes). For several BCAs, efficacy is substantially lower in the field than under the controlled conditions of the laboratory (Elmer and Reglinski 2006). A possible reason for poor efficacy in the field is that BCAs are often used in a similar manner as fungicides. In viticulture, for instance, BCAs are used at the four specific grape growth stages when fungicides are commonly applied: A, end of flowering (BBCH69 of Lorenz et al. 1995); B, pre-bunch closure (BBCH77); *C,* veraison (BBCH81); and D, pre-harvest

[1] A search in the Web of Science of "TS=((Botrytis) AND(biocontrol OR "biological control" OR BCA* OR "biological control agent*" OR (microbial AND antag*) OR biofungicide* OR "biological activity" OR micro-organism*))" yielded 2064 results from 1986 to 2019.

(BBCH<89) (González-Domínguez et al. 2019, see Chapter 2). This approach does not consider that BCAs are living organisms that dynamically interact with the target pathogen, the host plant, and the microbial communities on the host surfaces in a changing physical environment. Indeed, BCA establishment, growth on the host surfaces, and efficacy are strongly influenced by weather conditions, including temperature, relative humidity, wetness duration, and solar radiation (Elad and Freeman 2002; Kredics et al. 2003). The same is true for infection by *B. cinerea* (Holz et al. 2007). A successful integration of BCAs in a disease management strategy therefore requires an understanding of their ecological requirements so that they are used when environmental conditions are favorable.
Over the past 15 years, the use of BCAs to control *B. cinerea* has been reviewed by Abbey et al. (2018), Elad and Stewart (2007), Elmer and Reglinski (2006), Sharma et al. (2009), Jacometti et al. (2010), and Haidar et al. (2016). These reviews mainly focus on biocontrol mechanisms and the commercial implementation of BCAs, but they provide little information on how the environment affects BCA behavior and efficacy in the field, in greenhouses, or in storage facilities.
In this work, a systematic review was conducted with the aims of i) reviewing the information about how environmental conditions affect the fitness and efficacy of microorganisms for biocontrol of *B. cinerea*; ii) proposing the "environmental niche" approach for studying BCA-pathogen relationships; and iii) proposing future research directions for better use of BCAs in practical crop protection.

The systematic literature review

A literature search strategy was developed according to the principles of systematic literature review. A systematic review is a way to locate and assemble what is known from the literature, and to synthesize the research findings into an accessible format (Mulrow 1994). The use of a systematic approach reduces errors, limits researcher bias, and improves the communication of the information (Candel 2014). Systematic methods require the use of clear inclusion and exclusion criteria to select eligible literature in order to answer a specific research question.
The data collection process used in this review is schematically described in Figure 7.1. This systematic review was conducted in February 2019 with the digital bibliographical databases Scopus, Web of Science, and Google Scholar. Given the topic of the review (i.e., the influence of environmental conditions on the biocontrol of *B. cinerea* by microorganisms), the published papers to be considered

must have tested the BCA against *B. cinerea* in any crop and must have determined the effect of different environmental conditions on the fitness of the BCA and/or its efficacy against the target pathogen. To be included in the review, papers had to satisfy the following criteria: (i) the papers were written in English; (ii) the term *Botrytis* appeared in the title, abstract, and/or authors' keywords (in order to exclude papers in which BCAs were tested against a different pathogen or in which *B. cinerea* was only mentioned in the manuscript); (iii) different environmental conditions were considered (e.g., different temperature regimes); and (iv) effects on BCA fitness and/or efficacy were reported. Based on these criteria, specific queries were formulated to search academic articles, reviews, articles in press, and conference papers in the three digital bibliographical databases (Scopus, Web of Science, and Google Scholar). The search was restricted to titles, abstracts, and keywords in Scopus and Web of Science, and to titles in Google Scholar. The specific queries were:

i. Scopus, performed on 14 February 2019 and resulting in 253 publications: (TITLE-ABS-KEY((Botrytis) AND(biocontrol OR "biological control" OR BCA* OR "biological control agent*" OR "microbial AND antag*" OR biofungicide* OR "biological activity" OR micro-organism*) AND(temperature* OR "water availability" OR "relative humidity" OR pH OR "metal ion*" OR "water potential" OR "water activity" OR environment*))).
ii. Web of Science, performed on 13 February 2019 and resulting in 257 publications: (TS=((Botrytis) AND (biocontrol OR "biological control" OR BCA* OR "biological control agent*" OR (microbial AND antag*) OR biofungicide* OR "biological activity" OR micro-organism*) AND(temperature* OR "water availability" OR "relative humidity" OR pH OR "metal ion*" OR "water potential" OR "water activity" OR environment*))).
iii. Google Scholar, performed on 1 February 2019 and resulting in 373 publications: (allintitle: Botrytis "biological control" OR environment).

The papers obtained from the first search were merged and duplicates excluded, leading to 347 documents (Fig. 7.1). All of the abstracts in these documents were loaded into RefWorks (ProQuest, MI, USA) and read. Based on the inclusion criteria, the papers were included in the final list if their abstract included one or more of the following kinds of information: (i) the microorganism was considered as a BCA; (ii) BCA fitness and/or efficacy was investigated; and (iii) changing environmental conditions were considered. At the end of this step, 126 papers

were selected, the full papers were read, and the papers were selected again by using the same inclusion and exclusion criteria used for abstracts. This led to a selection of 51 papers. Finally, the references of these 51 papers were checked, and 3 additional papers were included based on the previously listed criteria, leading to a final database of 54 academic papers (Fig 7.1).

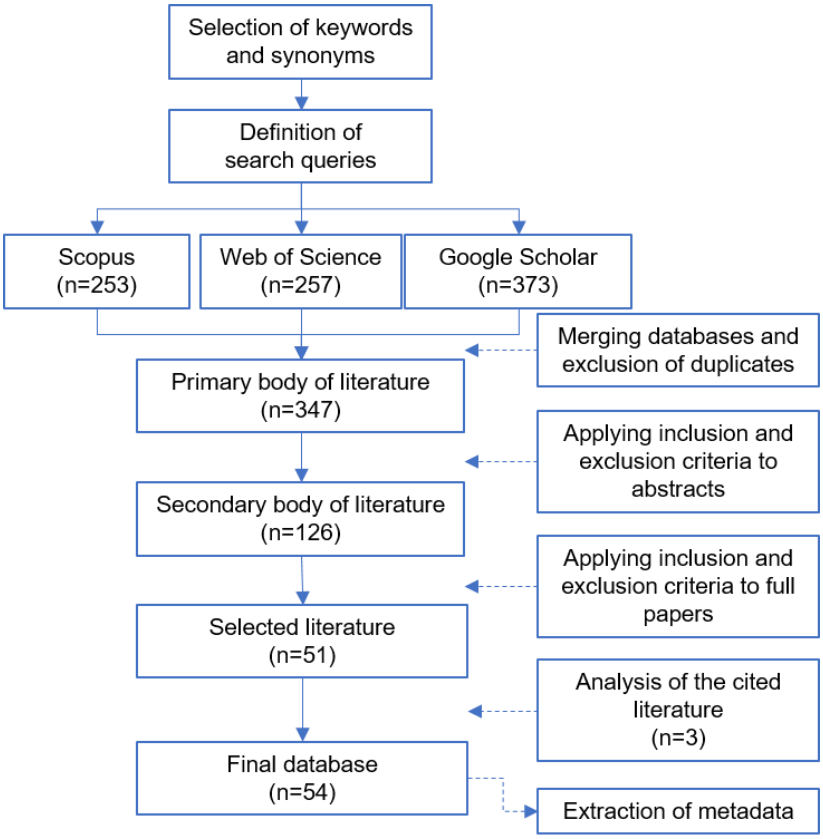

Figure 7.1. Flow of the systemic literature review (based on Candel 2014).

Main characteristics of the selected papers

Papers were analyzed based on the publication year, BCA, environmental conditions, type of study, and host crop.

Concerning the publication year, the number of papers addressing the general topic of *B. cinerea* biocontrol increased from the 1980s to the present time (data from Web of Science's search indicated in footnote 1) and especially over the last 10 years (orange line in Fig. 7.2). Papers relating these BCAs with the environmental conditions were published more frequently in the 1990s than in the following decades, i.e., only 1 or 2 papers were published per year in most years after 2003 (grey bars in Fig. 7.2).

Concerning the microorganisms, the selected papers investigated 42 species belonging to 27 genera as potential BCAs (Fig 7.3). Most of the potential BCAs are eukaryotes (76% of the cases, in which a "case" is a study in which a BCA was investigated in the selected papers), with the phylum Ascomycota (62%) more represented than Basidiomycota (14%) or others (Fig 7.3). The domain of Bacteria accounted for 24% of the cases, with microorganisms belonging to the phylum Firmicutes (14%) and Proteobacteria (10%, Fig 7.3). The Ascomyceta *Trichoderma* was the most studied (in 15 cases), especially the species *T. harzianum* (8 cases), followed by the genera *Candida* (9 cases) and *Clonostachys* (9 cases). *Rhodotorula* was the most studied genus of Basidiomycota, especially the species *R. glutinis* (5 cases). *Bacillus* (7 cases) and *Pseudomonas* (3 cases) were the most considered Bacteria (Fig. 7.3).

Concerning the type of study, 41% of the selected papers focused on the control of *B. cinerea* as a post-harvest pathogen, and all of these studies were conducted under laboratory conditions (Fig. 7.4). When the BCAs were used for the pre-harvest control of the pathogen, 31% of the studies were conducted only in the laboratory, 11% in both the laboratory and greenhouse, 9% in both the laboratory and field, and 6% in the laboratory, greenhouse, and field. In one case, BCA efficacy was studied only in the greenhouse (Fig. 7.4).

Regarding the host crop, because *B. cinerea* affects a wide range of crops (Jarvis 1977), 16 crops were considered, including horticultural crops, fruit trees, and ornamentals (Fig. 7.5). Thirty-one papers (47.7% of the total) focused on *B. cinerea* affecting fruit tree crops. In apple, kiwi, pear, and cherry, the effect of BCAs was only assessed in post-harvest, because *B. cinerea* is most damaging during fruit storage for these crops (Droby and Lichter 2007). Horticultural crops were considered in 28 papers (43.1% of the total papers), with strawberry and tomato being the most important. Ornamental crops were considered in only 6 cases (9.2%), specifically for the *B. cinerea* hosts geranium, cyclamen, and rose (Fig. 7.5).

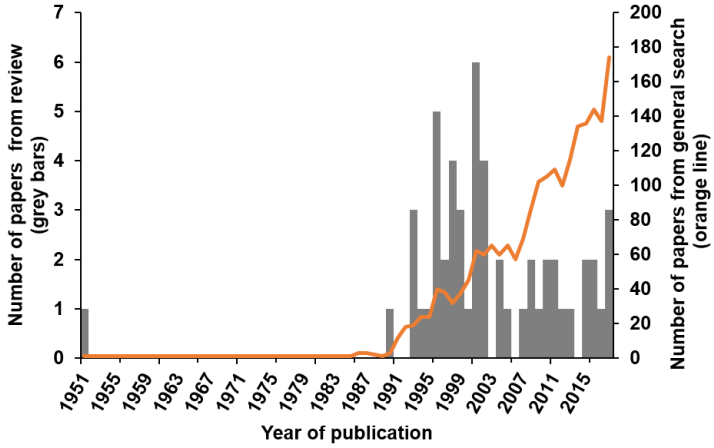

Figure 7.2. Number of papers published per year that evaluated the biocontrol of *Botrytis cinerea* in general (orange line) and the effect of environmental conditions on the fitness and/or efficacy of BCAs against *B. cinerea* in particular (grey bars). Data for the orange line were obtained by searching the Web of Science with the query "TS=((Botrytis) AND (biocontrol OR "biological control" OR BCA* OR "biological control agent*" OR (microbial AND antag*) OR biofungicide* OR "biological activity" OR micro-organism*))". Data for grey bars were obtained from the systematic literature review (final database of 54 papers, as indicated in Fig. 7.1).

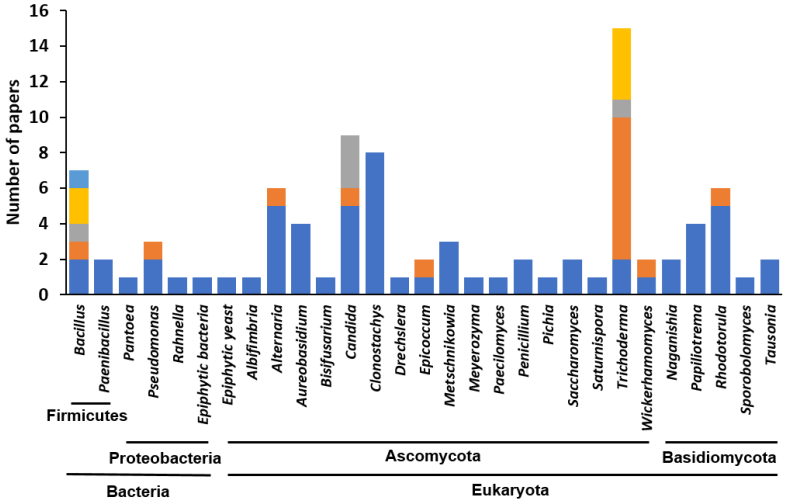

Figure 7.3. Number of papers that evaluated the influence of environmental conditions on the biocontrol of *Botrytis cinerea* per genus of BCA. The numbers of papers published for different species in each genus are indicated with different colors within the bars.

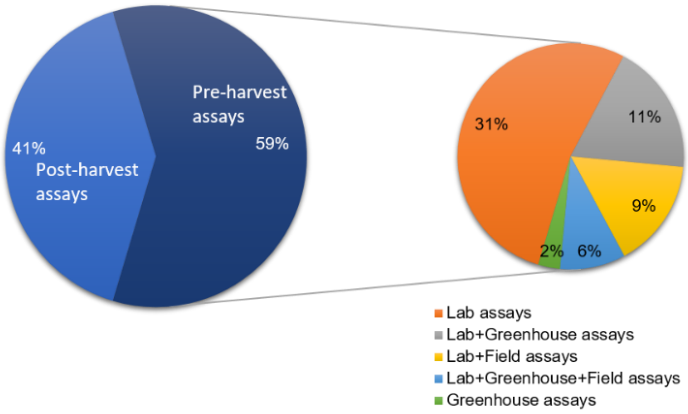

Figure 7.4. Percentages of the papers that evaluated the influence of environmental conditions on the biocontrol of *Botrytis cinerea* per type of assay.

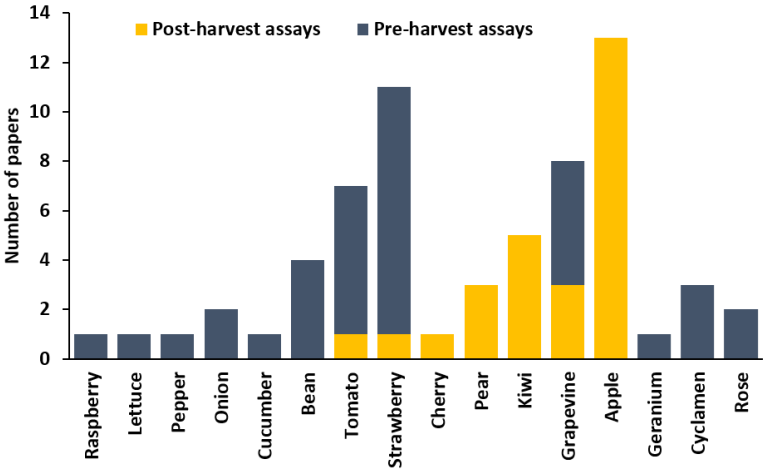

Figure 7.5. Number of papers that evaluated the influence of environmental conditions on the biocontrol of *Botrytis cinerea* per type of crop.

Effect of the environment on BCA fitness and efficacy

The selected papers assessed the effect of the environment on BCA fitness and/or efficacy against *B. cinerea* (Tables 7.1 and S7.1). For some BCAs, the effect of environmental conditions was investigated for fitness only (*Pantoea* and *Pichia*) or efficacy only (e.g., *Saccharomyces* or *Albifimbria*) (Table 7.1).

For fungal BCAs, fitness is considered to be the ability to grow in a given environment (Brasier 1999) as indicated by i) spore germination (germination rate and/or germ tube length); ii) mycelial growth (radial growth of colonies), and/or iii) population size (colony forming units, CFUs; or Optical Density, OD) (Table 7.1). For bacterial BCAs, fitness is considered to be the ability to replicate in a given environment (Wiser and Lenski 2015) and is studied by assessing population size (CFUs; most probable number, MPN; or OD). Efficacy of both fungal and bacterial BCAs is evaluated as the ability to reduce i) disease incidence and/or severity; ii) mycelial growth of *B. cinerea* on plant surfaces; iii) the germination of *B. cinerea* conidia; and/or iv) *B. cinerea* sporulation on the host surface.

Fitness was investigated for 19 of the 27 genera of BCAs (Table 7.1) and mainly in terms of CFUs (Table 7.1). Efficacy of BCAs against *B. cinerea* was evaluated for 24 of the 27 genera and mainly as a reduction of disease incidence in treated vs. untreated plant material (Table 7.1). The great majority of papers evaluated the effect of different temperatures, and half of them also evaluated the effect of humidity (Table 7.1). In these papers, humidity was evaluated as water activity (a_w), water potential (MPa), or relative humidity (RH); for our analysis, a_w and MPa values were converted into RH values as described by Troller (1983) and Köhl (2004), respectively. Only a few papers considered the effect of pH (Table 7.1), even though pH directly influences the fitness of BCAs and, as a consequence, their efficacy against *B. cinerea* (Teixido et al. 1998).

The next sections in this review consider the effects of environment on fitness and/or efficacy for the most studied BCA genera. BCAs are considered at the genus level and not as single species because little information exists at the species level. To increase the probability of obtaining consistent results, metadata were calculated only for those genera represented by at least 4 papers. The metadata summarize the available information for the effect of temperature (T), relative humidity (RH), and pH in terms of intervals investigated (e.g., 5°C intervals for temperature) and cardinal values, i.e., minimal, optimal, and maximal values for the dependent variable (e.g., spore germination or disease control). Cardinal values were estimated for fitness and efficacy as a whole, i.e., with no distinction among spore germination, mycelial growth, and population size for fitness, or among effects on disease incidence/severity, growth, germination, or sporulation

of *B. cinerea* on the host for efficacy. Cardinal values were also determined for *B. cinerea*.

The above metadata were used to determine the environmental niches for *Trichoderma* and *Candida* BCAs, and to compare their environmental niches with that of *B. cinerea*. Environmental niches are the environmental conditions necessary for the presence of a species and for the maintenance of its population (Chesson et al. 2001). In this work, environmental niches were defined considering temperature and humidity intervals in which the growth was null (no growth), minimal (<=20% of maximum growth), marginal (>20-50%), considerable (>50-80%), and maximal (>80%). A score was assigned to the above growth rates as follows: no growth=0; minimal=1; marginal=2; considerable=3; and maximal=4.

A first analysis was conducted to determine the temperature and RH combinations at which the growth rate of the BCA (*Trichoderma* or *Candida*) was higher than, equal to, or lower than the growth rate of *B. cinerea*. In the first step, values 1, 0, and -1 were assigned to these three conditions (higher than, equal to, and lower than, respectively) for temperature and RH, separately. In the second step, a matrix was developed in which rows were the values (1, 0, or -1) for RH, and columns were the values (1, 0, or -1) for temperature; in this matrix, cells are the sum of the values in rows and columns. Cells can then take the following values: 2, the growth of BCA is much higher than that of *B. cinerea* (BCA>>Bc); 1, BCA>Bc; 0, BCA=Bc; -1, BCA<Bc; and -2 (BCA<< Bc). In the third step, a color map was created in which the cell values are represented by colors: 2, dark green; 1, green; 0, yellow; -1, orange; -2, red (Fig. 7.7D and 7.7E).

A second analysis was conducted to determine the extent of environmental niche sharing by the BCA (*Trichoderma* or *Candida*) and the target organism (*B. cinerea*). In the first step of this analysis, the growth scores (from 0 to 4) of each BCA and of *B. cinerea* were multiplied. In the second step, a matrix was developed in which rows are the above products for RH, and columns are the products for T; in this matrix, cells are the products of the values in rows and columns. In the third step, a color map was created based on the matrix (Fig. 7.8A and 7.8B). The frequency of cells in which the product is >0 shows the extent of T and RH combinations in which the two microorganisms interact; the sum of the values in the cells provides information on the intensity of such an interaction.

Table 7.1. Environmental variables considered in studies of the fitness and/or efficacy of several biological control agents (BCAs) against *Botrytis cinerea*.

	Fitness			Efficacy (Reduction of)			
BCA	**Spore germination**	**Mycelial growth**	**Population size**	**Disease**	**Surface colonization**	**Germination**	**Sporulation**
Bacteria							
Bacillus	na[1]	na	T, pH	T, RH	pH	T	-
Paenibacillus	na	na	T, pH	T	-	-	-
Pantoea	na	na	T	-	-	-	-
Pseudomonas	na	na	T, pH	T	-	-	-
Rahnella	na	na	T	T	-	T	-
Eukaryota							
Albifimbria	-	-	-	T, RH	T, RH	-	-
Alternaria	T, RH	T	-	T, RH	T, RH	-	T, RH
Aureobasidium	-	-	T	T, RH	pH	T, RH	T, RH
Bisifusarium	-	-	-	T, RH	-	-	-
Candida	-	-	T, RH, pH	T	pH	-	-
Clonostachys	T, RH	T	T	T, RH	T, RH	-	T, RH
Drechslera	-	-	-	T, RH	T, RH	-	-
Epiccocum	-	-	-	T, RH	T, RH	-	T
Metschnikowia	-	-	T	T	T	-	-
Meyerozyma	-	-	T	T, RH	-	T, RH	-
Paecilomyces	-	T	-	-	-	-	-
Penicillium	T	-	-	T	-	-	-
Pichia	-	-	T	-	-	-	-
Saccharomyces	-	-	-	T	-	-	-
Saturnispora	-	-	-	T	-	-	-
Trichoderma	T	T	T, RH	T, RH	T, RH	T, RH	T, RH
Wickerhamomyces	-	-	T, RH pH	-	pH	-	-
Naganishia	-	-	-	T, RH	-	T, RH	T, RH
Papiliotrema	-	-	T	T	-	-	-
Rhodotorula	-	-	T, RH, pH	T	-	-	-
Sporobolomyces	-	-	-	T	-	-	-
Tausonia	-	-	T	T	-	-	-

[1] na: not applicable; T: temperature; RH: relative humidity; -: no information available in the meta-analysis

Fitness of BCAs

Information was available for 19 genera of BCAs (Table 7.1); Fig. 7.6 summarizes the environmental requirements for *B. cinerea* and for the 8 BCAs that were assessed in at least 4 of the papers in the final database. *Botrytis cinerea* develops at temperatures between 0 and 35°C, at RH values between 54 and 100%, and at pH values between 1 and 10, with optima at 20-25°C, 100% RH, and pH 3-7 (Jarvis 1977; Alam et al. 1996; Ciliberti et al. 2016).

BCAs were mostly studied in the range of 0 to 37°C, except that information on the effects of temperatures >25°C on *Trichoderma* exists only for other pathogens (Sutton and Peng 1993; Hjeljord et al. 2000; 2001; Fig. 7.6). All BCAs grew when temperatures were >5°C and <33°C, with the exception of *Bacillus* and *Clonostachys*, which grew at 37°C, i.e., the maximum tested (Köhl et al. 1999; Guetsky et al. 2001; Cota et al. 2008; Calvo et al. 2017). The overall temperature range for optimal growth of the BCAs was 18 to 32°C, which partially overlaps with the optimum for *B. cinerea* (20-25°C) (Fig. 7.6). The optimal temperatures for growth were between 18 and 23°C for *Trichoderma* and *Papiliotrema* (Roberts 1990; Elad and Kirshner 1993; O'Neill et al. 1996), between 20 and 25°C for *Candida* (Teixido et al. 1998), between 23 and 28°C for *Aureobasidium* (Lima et al. 1997), and between 25 and 30°C for *Bacillus, Alternaria*, *Clonostachys*, and *Rhodotorula* (Köhl et al. 1999; Zapata et al. 2001; Calvo et al. 2017; Fig. 7.6).

The effect of RH has been studied for *Alternaria*, *Trichoderma*, *Rhodotorula*, and *Candida* (Fig. 7.6). In general, fitness of both *B. cinerea* and the BCA increased when RH increased. *Rhodotorula* was able to grow at RH values from 94 to 100%, but data are lacking for its growth when RH is <94% (Zapata et al. 2001). *Candida* and *Trichoderma* grew under low RH conditions (>90% and 60%, respectively; O'Neill et al. 1996; Teixido et al. 1998), while *Alternaria* growth required RH values >95% (Köhl 2004) (Fig. 7.6). A relationship was detected between temperature and minimal humidity requirements (Teixido et al. 1998).

The effect of pH has been investigated for *Bacillus*, *Candida*, and *Rhodotorula*. *Bacillus* and *Candida* grew at pH 3 to 7, but they grew better at pH >5. *Rhodotorula* growth was not affected when pH ranged from 3 to 9.

Figure 7.7 shows the environmental niches for *B. cinerea* (Fig. 7.7A) (Thomas et al. 1988; Broome 1995; Williamson et al. 1995; Eden et al. 1996; Latorre and Rioja 2002; Lahlali et al. 2007; Ciliberti et al. 2016), *Trichoderma* (Fig. 7.7B) (Elad and Kirshner 1993; Pratella and Mari 1993; Sutton and Peng 1993; O'Neill et al. 1996; Hjeljord et al. 2000; 2001), and *Candida* (Fig. 7.7C) (Mercier and Wilson 1994; Mercier and Wilson 1995; Lima et al. 1997; Teixido et al. 1998; Nunes et al. 2002; Carbó et al. 2018a; 2018b).

Figures 7.7D and 7.7E indicate the combinations of temperature and RH conditions (environmental niches) at which the growth of the BCA (*Trichoderma* in Fig. 7.7D and *Candida* in Fig. 7.7E) is greater than, equal to, or less than that of *B. cinerea*. For instance, *B. cinerea* frequently prevails over *Trichoderma* when T<10°C regardless of RH and when T<26°C and RH<72% (Fig. 7.7D); under these conditions, *B. cinerea* is expected to be more competitive than *Trichoderma*. When T>10°C and RH>72%, the two microorganisms grow at similar rate or *Trichoderma* prevails over *B. cinerea*, especially when T>26°C and RH>72%; under the latter conditions, the BCA is expected to be more competitive in occupying the niches where it interacts with *B. cinerea* and to be more effective in disease control. In the second case (Fig. 7.7E), *B. cinerea* mostly prevails over *Candida* under most temperature and RH conditions due to the strong limiting effect of RH on *Candida* growth. Teixido et al. (1998) reported that the minimum a_w for the growth of *Candida sake* was 0.9 at the optimum temperatures of 20–25 °C, and that that the minimum a_w was higher at lower or higher temperatures.
B. cinerea shares a wider ecological niche with *Trichoderma* (Fig. 7.8A) than with *Candida* (Fig. 7.8B). The frequency of niche sharing is 59 and 18% for *Trichoderma* and *Candida*, respectively, and the intensity of interaction is 573 and 161 for the two BCAs, respectively. These results suggest that *Trichoderma* has the potential to compete with the target pathogen under a wider range of environmental conditions than *Candida*, and that *Trichoderma* might provide disease control under changing environmental conditions in the field.

Efficacy of BCAs against *B. cinerea*

Information on efficacy against *B. cinerea* is available for 24 genera (Table 7.1); Figure 7.9 summarizes the environmental conditions of the 8 BCAs that were assessed in at least 4 of the papers in the final database. The efficacy of *Bacillus* has been tested at temperatures ranging from 0 to 37°C; it was not effective at <5°C and was most effective at 20-25°C (Mari et al. 1996; Guetsky et al. 2001). Similar optimal temperatures have been reported for *Alternaria*, *Aureobasidium*, *Clonostachys*, and *Trichoderma* (Hannusch and Boland 1996; Dik et al. 1999). Information for *Candida*, *Papiliotrema*, and *Rhodotorula* is available for a narrower temperature range (Fig. 7.9). For instance, disease suppression by *C. sake* has been studied only at 0 to 10°C (Cook et al. 1999). The efficacy was highest at 5 to 15°C for *Papiliotrema* and at 10°C for *Rhodotorula* (Roberts 1990; Helbig 2002).
Information is not available for the effect of RH on the efficacy of *Candida*, *Papiliotrema*, or *Rhodotorula*. For the other BCAs, the optimal levels of RH are ≥90% (Fig. 7.9). For *Aureobasidium* and *Trichoderma,* no efficacy was evident at

RH<90% and <60%, respectively (Dik et al. 1999), while the effect of RH<90% is not known for *Clonostachys* (Hannusch and Boland 1996).
pH values between 4-6 did not influence the efficacy of *Candida* (Junior et al. 2016) or *Aureobasidium* (Parafati et al. 2015), while a pH of 6 was the optimum for *Bacillus* (Wang et al. 2013).

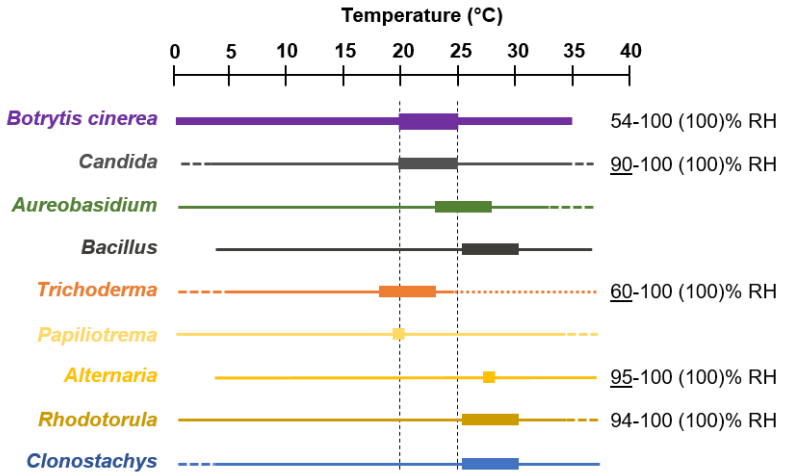

Figure 7.6. Environmental requirements for the fitness of the main BCAs against *Botrytis cinerea*. Thin lines indicate the temperature range; thick lines indicate optimal temperatures; dashed lines indicate temperatures that are known not to support growth of the BCA; dotted lines indicate temperatures tested for the BCA without regard to *B. cinerea*. The vertical lines indicate the optimal temperature range for *B. cinerea*. The relative humidity (RH) conditions are shown as the range and optimal values (brackets); underlined values indicate values that do not support the growth of the BCA.

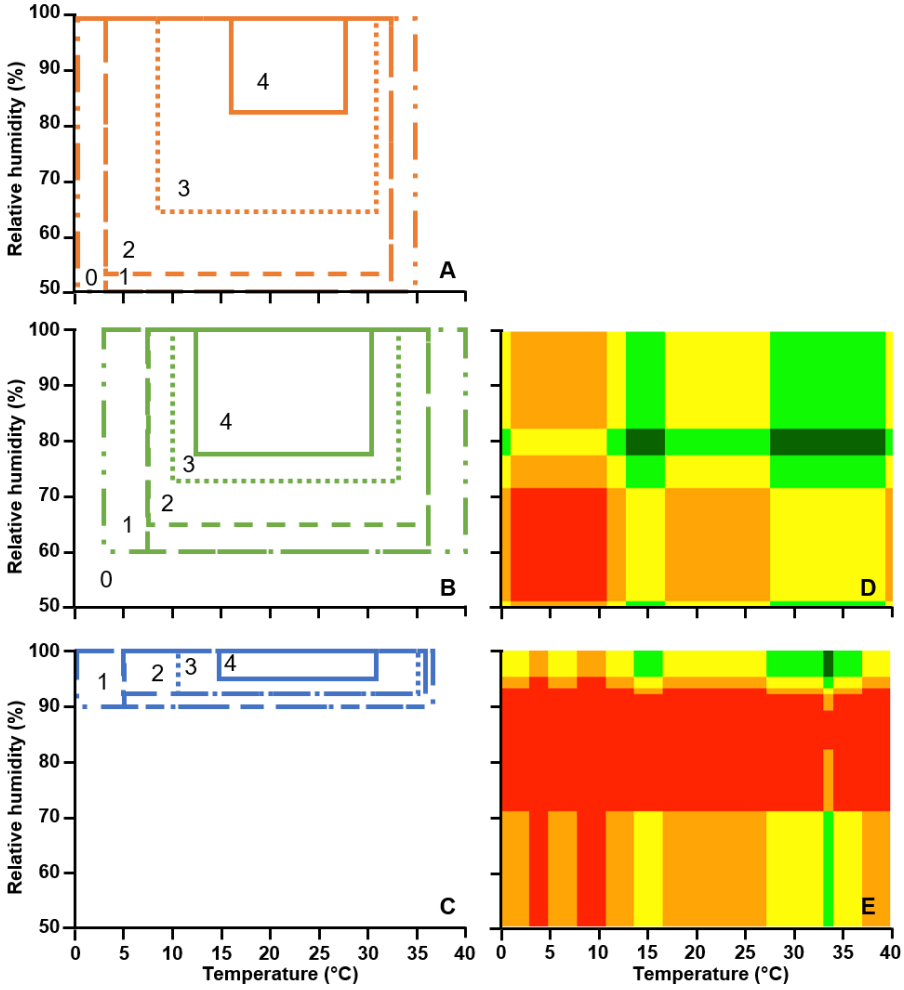

Figure 7.7. Environmental niches for *Botrytis cinerea* (**A**), *Trichoderma* (**B**), and *Candida* (**C**) (based on Magnuson et al. 1979). Environmental niches are shown as temperature and relative humidity ranges for no (0), minimal (1), marginal (2), considerable (3), and maximal (4) growth. **D** and **E** show the combinations of environmental conditions in which the growth of the BCA is higher than (in green and dark green), equal to (yellow), or lower than (orange and red) the growth of *B. cinerea*.

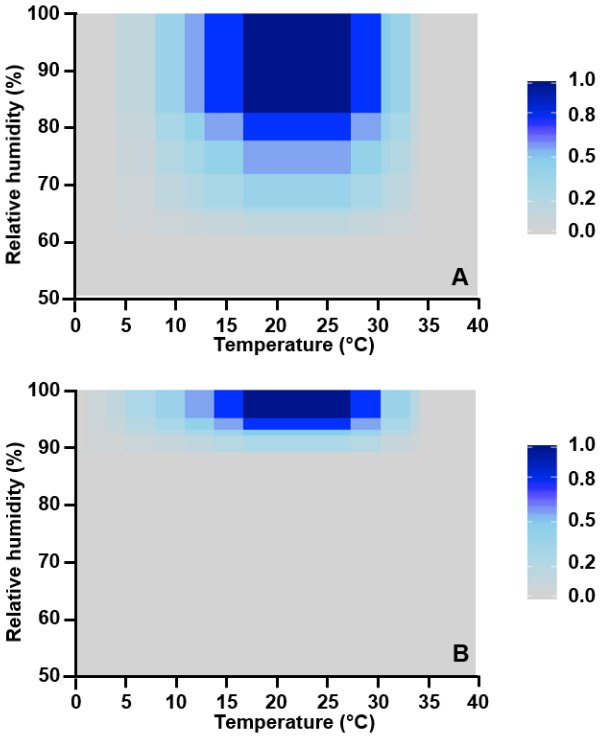

Figure 7.8. Extent of environmental niche sharing by *Botrytis cinerea* and the BCAs *Trichoderma* (**A**) or *Candida* (**B**). Each map is the product matrix of the environmental niches of the pathogen and BCA. Dark blue (1.0) indicates complete overlap of environmental niches; light blue (0.2) indicates weak overlap of environmental niches; and grey indicates environments that do not support the growth of the pathogen or the BCA.

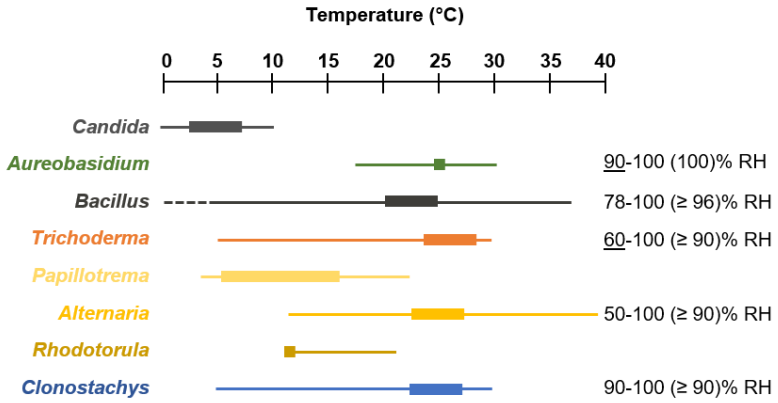

Figure 7.9. Environmental requirements for the effectiveness of the main BCAs against *Botrytis cinerea*. Thin lines indicate the temperature range; thick lines indicate optimal temperatures; dashed lines indicate temperatures that are known not to support growth of the BCA. The relative humidity (RH) conditions are shown as the range and optimal values (brackets); underlined values indicate values that do not to support the growth of the BCA.

Conclusions and future prospects

The Directive 128/2009/EC on the Sustainable Use of Pesticides (SUD) makes low pesticide-input control strategies mandatory in the EU. The SUD promotes the use of non-chemical methods as alternatives to pesticides, whenever possible. Non-chemical methods include BCAs, which have been intensively studied in recent decades and have been used as active ingredients in several currently available products (Haidar et al. 2016). Nevertheless, the biocontrol of plant diseases remains challenging, and the farmer reliance on BCAs as a valid alternative to chemical fungicides remains marginal (Tracy 2014). The failure of many farmers to adopt BCAs for disease control can be explained by the inconsistent efficacy of biocontrol, which in turn may be related to changing environmental conditions at the time when BCAs are applied in the field or in storage (Haidar et al. 2016). Therefore, insufficient knowledge about the environmental effects on the BCA fitness and efficacy may be a bottleneck for increasing BCA use by farmers.

Hundreds of papers have been published on the biocontrol of *B. cinerea*, and the subject has also been reviewed several times (Elmer and Reglinski 2006; Elad and Stewart 2007; Sharma et al. 2009; Jacometti et al. 2010; Haidar et al. 2016; Abbey et al. 2018). None of these reviews, however, provides comprehensive information on how the environment affects BCA fitness and efficacy. In the present systematic literature review, only 54 papers (from an initial number of 347) were selected for analysis of the effect of environmental conditions on the fitness and efficacy of

BCAs for *B. cinerea* control. These papers consider 27 genera of fungi and bacteria, and only a few species of BCAs have been considered in multiple studies. For this reason, the BCAs were meta-analyzed at the genus level instead of at the species level. This may have introduced an error due to the intra-genus variability in environmental responses. However, consistency was observed among species belonging to the same genus (i.e., they have similar responses to different environmental conditions; *data not shown*). For example, *Trichoderma viride* (Sutton and Peng 1993), *T. harzianum* (Hjeljord et al. 2000), and *T. atroviride* (Hjeljord et al. 2001) have the same temperature range (i.e., 20-25°C) for optimal germination of conidia. Similarly, populations of *Bacillus amyloliquefaciens* (Calvo et al. 2017), *B. subtilis* (Pershakova et al. 2018), and *B. mycoides* (Guetsky et al. 2001) all have an optimal temperature range from 25 to 30°C.

The present literature review indicates that information about the effects of temperature, RH, and pH on the fitness and efficacy BCAs is quite limited. Frequently, studies have considered only one measure of fitness or efficacy and only one environmental variable (Table 7.1). This is also true for studies of some popular BCA genera, such as *Candida* and *Saccharomyces*. Although more information exists for *Trichoderma*, most *Trichoderma* papers provide only one measure of fitness. This insufficient databased is surprising because knowledge of the environmental requirements for BCA establishment, colonization, and efficacy should be the foundation for the development of efficient disease control strategies.

The absence of the term “Botrytis” in the title, summary, or keywords of the papers selected through the systematic literature review may have excluded some papers that assessed the effect of environmental conditions on the fitness of BCAs in general or on the control of pathogens other than *B. cinerea*. Although these papers might have been included in our meta-analysis, they were not included because when BCAs are used against a target pathogen other than *B. cinerea*, the context may be very different. For instance, *Trichoderma*, *Pseudomonas*, and *Bacillus* have been used for the biocontrol of various soilborne fungal diseases (Heydari and Pessarakli 2010). In this work, the possibility of failing to obtain useful information by excluding papers that do not directly deal with *B. cinerea* was partially addressed by checking the references of selected papers and by reviewing them, when appropriate.

The concept of environmental niches proposed in the present chapter may help researchers identify those BCAs that occupy (or partially occupy) the same niche as the target pathogen (*B. cinerea* in this review); such BCA may therefore have an increased probability of growing under the same environmental conditions under which *B. cinerea* grows; this may lead to greater interaction (which can be

parasitism, competition or antagonism, depending on the BCAs' characteristics) between the microorganisms and therefore to higher efficacy of the BCA. The environmental niches for *B. cinerea*, *Trichoderma*, and *Candida* were described by using the metadata obtained in the systematic literature review, and they could probably be improved by consideration of additional literature or by conducting additional research. The intention here is not to propose definitive niches for these microorganisms, but to show how the environmental niche approach works and to discuss its possible uses.

Environmental niches can be useful for screening new BCAs based on their ability to occupy the same environmental niche as the target pathogen. Environmental niches can also help in the selection of the BCAs to be used in the field. For instance, if the pathogen niche is not completely covered by one BCA, a second BCA could be combined to extend the environmental niche occupied by both BCAs. Furthermore, when different BCAs are available to control a pathogen, the selection of the BCA to be used in a specific field application should include consideration of the weather conditions at application and those forecast in the days following application so as to increase the probability that the selected BCA will occupy the target's environmental niche.

Environmental niches could also be a starting point for the development of dynamic, weather-driven models for the prediction of BCA effectiveness. Although mathematical models of the BCA-pathogen-host plant interactions have been developed (Jeger et al. 2009; Cunniffe and Gilligan 2011; Xu et al. 2011), the models focus on the mechanisms of biocontrol without regard for the environment. A number of weather-driven models are available for pathogens, including *B. cinerea* (Xu et al. 2000; González-Domínguez et al. 2015), and BCAs could be incorporated into these models. At present, no weather-driven models are available for BCAs of *B.cinerea*.

New models addressing the effect of environment on BCA-plant pathogen systems are needed. Development of these models will require a deeper knowledge of the biology and epidemiology of the BCAs and of how the environment affects their fitness and efficacy against *B. cinerea*. Because obtaining the needed information could be challenging, priority should be given to the most-studied species of BCAs and for those included in commercial products. Once new information becomes available, models accounting for both biocontrol mechanisms and environmental conditions could be developed, which may help enhance the biocontrol *B. cinerea*.

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Supplementary material

Table S7.1. Environmental variables considered in the referenced studies in relation to the fitness and/or efficacy of several biological control agents (BCAs) against *Botrytis cinerea*.

	Fitness			Efficacy (Reduction of)			
BCA	**Spore germination**	**Mycelial growth**	**Population size**	**Disease**	**Surface colonization**	**Germination**	**Sporulation**
Bacteria							
Bacillus amyloliquefaciens	na[1]	na	Calvo 2017$^{T, pH}$	Mari 1996^{T}	-	-	-
B. dendroides	na	na	-	Wood 1951^{T}	-	-	-
B. coagulans	na	na	-	-	Wang 2013^{pH}	-	-
B. mycoides	na	na	Guetsky 2001^{T}	Guetsky 2001$^{T, RH}$	-	Guetsky 2001^{T}	-
B. subtilis	na	na	Pershakova 2018^{T}	Xu 2010^{T}	-	-	-
Paenibacillus polymyxa	na	na	Gu 2013$^{T, pH}$	Helbig 2001^{T}	-	-	-
Pantoea agglomerans	na	na	Nunes 2002^{T}	-	-	-	-
Pseudomonas fluorescens	na	na	Bisutti 2015$^{T, pH}$	Wood 1951^{T}	-	-	-
P. syringae	na	na	-	Janisiewicz 1992^{T}	-	-	-
Rahnella aquatilis	na	na	Calvo 2007^{T}	Calvo 2007^{T}	-	Calvo 2007^{T}	-
Eukaryota							
Albifimbria verrucaria	-	-	-	Hannusch 1996$^{T, RH}$	Hannusch 1996$^{T, RH}$	-	-
Alternaria atra	Köhl 1999^{T} Köhl 2004$^{T, RH}$	Köhl 1999^{T} Kessel 2005^{T}	-	Köhl 1999^{T}, Köhl 2004^{T,RH}, Nicot 2002$^{T, RH}$	-	-	Schoene 2002^{T,RH}
A. alternata	-		-	Hannusch 1996$^{T, RH}$	Hannusch 1996$^{T, RH}$	-	-

	Fitness			Efficacy (Reduction of)			
BCA	**Spore germination**	**Mycelial growth**	**Population size**	**Disease**	**Surface colonization**	**Germination**	**Sporulation**
Aureobasidium pullulans	-	-	Lima 1997[T] Vero 2009[T]	Dik 1999[T, RH]	Parafati 2015[pH]	Dik 1999[T, RH]	Dik 1999[T, RH]
Bisifusarium dimerum	-	Kessel 2005[T]	-	Nicot 2002[T, RH]	-	-	-
Candida sake	-	-	Teixido 1998[T, RH, pH] Carbò 2018a[T, RH] Carbò 2018b[T, RH, pH]	Cook 1999[T]	-	-	-
C. zemplinina	-	-	Nunes 2002[T] -	-	Junior 2016[pH]	-	-
C. oleophila	-	-	Lima 1997[T] Mercier 1994[T] Mercier 1995[RH]	-	-	-	-
Clonostachys rosea	Köhl 1999[T] Sutton 1993[T] Yu 1998[T, RH]	Köhl 1999[T] Pratella 1993[T] Cota 2008[T]	Morandi 2001[T]	Köhl 1999[T] Sutton 1993[T] Yu 1998[T, RH] Hannusch 1996[T, RH]	Hannusch 1996[T, RH]	-	Szandala 2001[T, RH] Yu 1998[T, RH]
Drechslera spp.	-	-	-	Hannusch 1996[T, RH]	Hannusch 1996[T, RH]	-	-
Epiccocum purpurascens	-	-	-	Hannusch 1996[T, RH]	Hannusch 1996[T, RH]	-	-
E. nigrum	-	-	-	-	-	-	Szandala 2001[T]
Metschnikowia pulcherrima	-	-	Piano 1997[T]	Cook 1999[T]	Parafati 2015[T]	-	-
Meyerozyma guilliermondii	-	-	Guetsky 2001[T]	Guetsky 2001[T, RH]	-	Guetsky 2001[T, RH]	-
Paecilomyces variotii	-	Pratella 1993[T]	-	-	-	-	-

	Fitness			Efficacy (Reduction of)			
BCA	**Spore germination**	**Mycelial growth**	**Population size**	**Disease**	**Surface colonization**	**Germination**	**Sporulation**
Penicillium spp.	Sutton 1993[T]	-	-	Sutton 1993[T] Wood 1951[T]	-	-	-
Pichia membranefaciens	-	-	Qin 2004[T]	-	-	-	-
Saccharomyces cerevisiae	-	-	-	Filonow 1998[T]	-	-	-
Saturnispora diversa	-	-	-	Li 2016[T]	-	-	-
Trichoderma spp.	-	-	-	Eden 1996[T]	-	-	-
T. atroviride	Hjeljord 2001[T]	-	-	Xu 2010[T]	-	-	-
T. harzianum	Hjeljord 2000[T]	Pratella 1993[T]	Elad 1993[T, RH] O'Neill 1996[T, RH]	O'Neill 1996[T, RH] Xu 2010[T] Dik 1999[T, RH] Elad 1993[T, RH]	-	Dik 1999[T, RH] Hjeljord 2001[T]	Szandala 2001[T, RH] Dik 1999[T, RH] Hjeljord 2001[T]
T. viride	Sutton 1993[T]	Pratella 1993[T]	-	Sutton 1993[T] Wood 1951[T] Hannusch 1996[T, RH]	Hannusch 1996[T, RH]	-	-
Wickerhamomyces anomalus	-	-	-	-	Parafati 2015[pH]	-	-
W. onychis	-	-	Zapata 2011[T, RH, pH]	-	-	-	-
Naganishia albida	-	-	-	Helbig 2002[T] Dik 1999[T, RH]	-	Dik 1999[T, RH]	Dik 1999[T,RH]

	Fitness			Efficacy (Reduction of)			
BCA	**Spore germination**	**Mycelial growth**	**Population size**	**Disease**	**Surface colonization**	**Germination**	**Sporulation**
Papiliotrema laurentii	-	-	Roberts 1990[T] Lima 1998[T] Qin 2004[T]	Filonow 1998[T] Roberts 1990[T]	-	-	-
Rhodotorula glutinis	-	-	Lima 1998[T] Qin 2004[T] Zhang 2008[T] Zapata 2011[T, RH, pH]	Helbig 2001[T]	-	-	-
R. mucilaginosa	-	-	Li 2011[T]	-	-	-	-
Sporobolomyces roseus	-	-	-	Filonow 1998[T]	-	-	-
Tausonia pullulans	-	-	Qin 2004[T]	Qin 2004[T]	-	-	-
Epiphytic bacteria	-	-	-	Sobiczewski 1999[T]	-	-	-
Epiphytic yeasts	-	-	-	-	-	Vargas 2012[T]	-

[1] na: not applicable; T: temperature; RH: relative humidity; -: no information available in the meta-analysis

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Chapter 8

Chapter 8

A generic model accounting for the interactions among pathogens, host plants, biocontrol agents, and the environment, with parametrization for *Botrytis cinerea* on grapevines[1]

Abstract

Although the use of biocontrol agents (BCAs) to manage plant pathogens has emerged as a sustainable means for disease control, the global reliance on their use remains relatively insignificant and the factors influencing their efficacy remain unclear.

In this work, we further developed an existing generic model for biocontrol of foliar diseases, and we parametrized the model for the *Botrytis cinerea*–grapevine pathosystem. The model was operated under three climate types to study the combined effects on BCA efficacy of four factors: i) BCA mechanism of action; ii) timing of BCA application with respect to timing of pathogen infection (preventative vs. curative); iii) temperature and moisture requirements for BCA growth; and iv) BCA survival capability.

All four factors affected biocontrol efficacy, but factors iii and iv accounted for >90% of the variation in model simulations. In other words, the most important factors affecting BCA efficacy were those related to environmental conditions.

These findings indicate that the environmental responses of BCAs should be considered during their selection, BCA survival capability should be considered during both selection and formulation, and weather conditions and forecasts should be considered at the time of BCA application in the field.

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Introduction

Biocontrol of plant pathogens has emerged as a sustainable method of disease management and as a viable way to reduce the application of chemicals in agriculture (Elad and Freeman 2002; Elmer et al. 2005; Harman 2000; Tracy 2014). The reasons for increasing restrictions on the use of chemicals and for increasing interest in biocontrol include the negative effects of chemicals on human health and the environment (Alavanja et al. 2004; Epstein 2014), pathogen-acquired resistance to commonly applied chemicals, and the lack of replacement products (Hahn 2014). Biocontrol involves the use of fungi, bacteria, yeasts, or viruses (together referred to as biocontrol agents or BCAs) that may suppress plant pathogens via competition for nutrients or space, antibiosis, parasitism, and induced host plant resistance (Elad and Freeman 2002).

Despite the extensive research on biocontrol and the potential of using BCAs as alternatives to chemicals, the global reliance on BCA use remains relatively insignificant (Tracy 2014). Many BCAs have been reported to suppress plant pathogens under controlled conditions in laboratories and greenhouses, but only a few have performed consistently in the field (Guetsky et al. 2001; Paulitz and Belanger 2001). A possible reason for the lack of success of BCAs in the field is that they are often used in a similar manner as fungicides, even though the processes influencing the efficacy of BCAs are complex (Rosenheim et al. 1995). The complexity is not surprising because BCAs are living organisms that dynamically interact with the target pathogen, the host plant, the microbial communities in the phyllosphere, and the physical environment (Fedele et al. 2019a, see Chapter 7). Fluctuating environmental conditions in the field influence BCA survival, establishment, growth, and activity (Elad and Freeman 2002; Kredics et al. 2003; Xu et al. 2010). Although temperature and humidity have been evaluated as key factors affecting BCA efficacy in some studies (Dik and Elad 1999; Elad et al. 1993; Fedele et al. 2019a, see Chapter 7; Hannusch and Boland 1996; Jackson et al. 1991; Mitchell et al. 1987; Kredics et al. 2003), the complex relationships between BCAs and the environment remain difficult to predict and manage (Deacon and Berry 1993; Whipps 1997).

Mathematical models have been used to study disease epidemics in relation to BCA dynamics. Some models focus on the relationship between BCA dose and pathogen infection (Cabrefiga and Montesinos 2005; Johnson 1994; Montesinos and Bonaterra 1996; Smith et al. 1997), while others consider more complex interactions (Cunniffe and Gilligan 2011; Knudsen and Hudler 1987; Kessel et al. 2005). Jeger et al. (2009) developed a mean-field deterministic model that is able to predict the likelihood of successful control of foliar diseases by a single BCA in relation to the biocontrol mechanisms involved. The latter model is

a standard susceptible-infected-removed (SIR) model, in which host–pathogen dynamics are coupled with pathogen–BCA dynamics through four biocontrol mechanisms: mycoparasitism, competition, antibiosis, and induced plant host resistance. Improved versions of this model were subsequently proposed to compare the effects of using a single BCA with two biocontrol mechanisms (Xu et al. 2010) vs. the combined use of two BCAs, each with an individual mechanism (Xu et al. 2011), or the effects of constant vs. fluctuating temperatures on biocontrol efficacy (Xu and Jeger 2013). The latter study revealed that the dynamics of biocontrol differed greatly under constant vs. fluctuating temperatures and stressed the importance of characterizing biocontrol activity in relation to environmental conditions and disease development.

In the current research, we enlarged the model proposed by Jeger et al. (2009) by including i) the effect of environmental conditions on the interactions between the pathogen and BCA and ii) the dynamics of host growth and senescence. The proposed model structure is generic and could be applied to various pathosystems and several pathogen–BCA interactions. We also parametrized the model for the *Botrytis cinerea*–grapevine pathosystem. *Botrytis cinerea* is the causal agent of Botrytis bunch rot (BBR), a serious disease that damages all grapevine organs and especially bunches, resulting in substantial losses of quantity and quality (Elad et al. 2016; Jarvis 1977; Williamson et al. 2007). We then operated the model under three climate types to determine whether the use of a specific BCA is more likely to result in effective biocontrol of *B. cinerea* depending on its adaptation to fluctuating conditions of temperature and relative humidity. In the following sections, we describe the model, its parametrization for the BBR case-study (i.e., Botrytis bunch rot in grapes, caused by *Botrytis cinerea*), and iii) model simulations for different BCAs under different climate types.

Model description

The model is based on the generic model developed by Jeger et al. (2009) and further revised by Xu et al. (2010). In this model, a classic susceptible-infected-removed (SIR) model for host–pathogen dynamics (Hethcote 1989) is combined with a model for pathogen–BCA dynamics. The modified model was developed by using a system dynamics approach (Leffelaar and Ferrari 1989) in which the system (consisting of the plant, the pathogen, the BCA, and the environment) is described by state variables, which represent plant tissue categories in relation to the pathogen–BCA interaction. The system moves from one state variable to another by mean of fluxes, which are regulated by rate variables (or rates). Rates depend on the characteristics of the pathogen, host plant, and BCA, and may also

be influenced by the weather conditions that affect the processes underlying the dynamics of both the pathogen (i.e., infection and infectiousness) and the BCA (i.e., growth and survival capability). The effect of external variables on processes is accounted for by driving functions (i.e., temperature, relative humidity, and moisture duration).

The model is generic and can be operated for fungal pathogens of aerial plant parts (e.g., leaves and fruits) and for BCAs with different mechanisms of action (MOA) including competition with the pathogen for space and nutrients, direct activity on the pathogen through antibiosis or mycoparasitism, and induced resistance in the plant. These are the main MOA of the currently used BCAs (Elad and Freeman 2002). The model works with a time step of 1 day.

The model was developed by using the software STELLA® (abbreviation of Systems Thinking, Experimental Learning Laboratory with Animation; 1.6.1. version; 2018), a visual programming language for system dynamics modelling. The model was diagrammed (Fig. 8.1) by using the graphic representation of Forrester (1961), which combines state variables (rectangles), flows (solid arrows), rates (valves), parameters and coefficients (circles), and numerical relationships (dashed arrows). Acronyms for state variables, rates, driving variables, and parameters are explained in Table 8.1.

Table 8.1. List of state variables, rates, driving variables, and parameters used in the model.

Symbol	Meaning of symbol	Dimension
K	Total surface area in the system	[1]
HS	Healthy-susceptible tissue	[N]
I	Affected by pathogen and infectious tissue	[N]
R	Affected by pathogen and removed tissue	[N]
H_r	Healthy and resistant tissue	[N]
H_b	Healthy and BCA colonized tissue	[N]
I_b	Infectious and BCA colonized tissue	[N]
R_b	Removed and BCA colonized tissue	[N]
BSUM	Total of BCA colonized tissue	[N]
RG	Rate of growth	$[N.T^{-1}]$
RS	Rate of senescence	$[N.T^{-1}]$
STARTP	Initial inflow of the pathogen into the system	$[N.T^{-1}]$
RI	Rate of infection	$[N.T^{-1}]$
RR	Rate of removal	$[N.T^{-1}]$

STARTB	Initial inflow of the BCA into the system	[N.T^{-1}]
RPIN	Rate of daily pathogen inflow	[N.T^{-1}]
RBIN	Rate of daily BCA inflow	[N.T^{-1}]
RRES	Rate of induction of resistance by BCA	[N.T^{-1}]
RSUS	Rate of change from H_r to HS tissue	[N.T^{-1}]
RCOLH$_r$	Rate of BCA colonization for H_r	[N.T^{-1}]
RCOLH	Rate of BCA colonization for HS	[N.T^{-1}]
RCOLI	Rate of BCA colonization for I tissue	[N.T^{-1}]
RCOLR	Rate of BCA colonization for R tissue	[N.T^{-1}]
GRO	Rate of BCA growth under fluctuating temperature and moisture	[N.T^{-1}]
BMORH	Rate of BCA mortality for the H_b tissue	[N.T^{-1}]
BMORI	Rate of BCA mortality for the I_b tissue	[N.T^{-1}]
BMORR	Rate of BCA mortality for the R_b tissue	[N.T^{-1}]
RAUDPC	Rate of AUDPC calculation	[N.T^{-1}]
RRG	Relative rate of growth	[N.N^{-1}.T^{-1}]
RRS	Relative rate of senescence	[N.N^{-1}.T^{-1}]
b	Relative rate of infection	[N.N^{-1}.T^{-1}]
h	Relative rate of change from I to R tissue	[N.N^{-1}.T^{-1}]
c_0	Relative rate of change from HS to H_r tissue	[N.N^{-1}.T^{-1}]
e	Relative rate of change from H_r to HS tissue	[N.N^{-1}.T^{-1}]
c_1	Relative rate of change from HS to H_b tissue	[N.N^{-1}.T^{-1}]
c_2	Relative rate of change from I to I_b and from R to R_b tissue	[N.N^{-1}.T^{-1}]
f	Relative rate of BCA mortality	[N.N^{-1}.T^{-1}]
COFR	Correction factor for occupied tissue	[1]
COFRH$_r$	Correction factor for H_r tissue	[1]
COFRI	Correction factor for I tissue	[1]
COFRR	Correction factor for R tissue	[1]
PDUR	Duration of mobilization of pathogen inoculum	[T]
PIN	Day of the first seasonal infection	[T]
BDUR	Duration of mobilization of BCA inoculum BDUR	[T]
BIN	Day of the BCA application	[T]
T	Driving function for daily temperature	[1]

MD	Driving function for daily moisture duration	[1]
RH	Driving function for daily relative humidity	[1]
AUDPC	Area under disease progress curve	[1]
POCC	Total K units occupied by the pathogen	[N]
EFF	Overall BCA efficacy	[1]
C	Relative contribution of competition	[1]
A	Relative contribution of antibiosis	[1]
IR	Relative contribution of induced resistance	[1]
P	Relative contribution of mycoparasitism	[1]

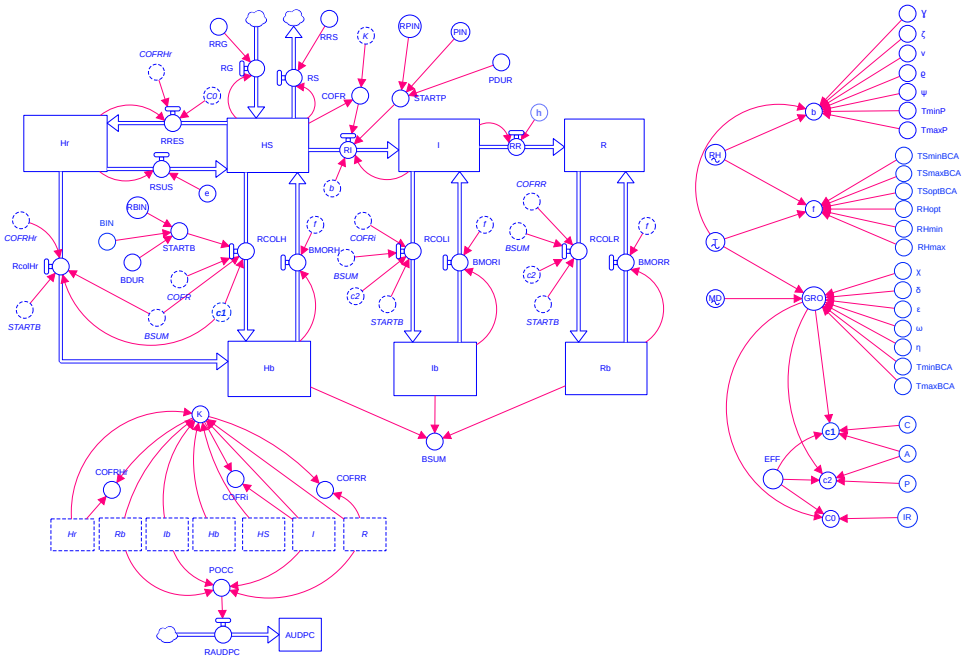

Figure 8.1. Model flowchart in which the state variables are host tissue categories that change according to the interactions among the pathogen, BCA, and the environment. The diagram uses the symbols developed by Forrester (1961). The core of the model is based on a classic susceptible-infected-removed (SIR) model, with tissue evolving from healthy-susceptible (HS) to infectious (I), and removed (R). The rate of infection of tissue (RI) depends on primary (STARTP) and secondary infections (I). The rate of resistance induction by a BCA (RRES) depends on BCA application (STARTB) and the total amount of healthy-susceptible tissue (HS). The rates of BCA colonization (RCOLH, RCOLI, RCOLR, and $RCOLH_r$) depend on BCA application (STARTB) and the total amount of colonized tissue (BSUM). The structure incorporates host growth (RG) and physiological senescence (RS). Symbols for state variables, rates, and parameters are explained in Table 8.1.

State variables and connecting flows

The site of the system consists of K units of plant tissue that can be potentially occupied (i.e., affected) by the pathogen during the epidemic. The K units represent the state variables of the model, and belong to one of the following non-overlapping categories of tissue: i) healthy and susceptible to infection (HS); ii) affected by the pathogen and infectious, i.e., can generate new, secondary infections (I); iii) affected by the pathogen and removed, i.e., no longer infectious (R); iv) healthy and colonized by the BCA, i.e., resistant to infection by the pathogen (H_r); v) healthy and colonized by the BCA, i.e., which is protected from

the pathogen (H_b); vi) infectious and colonized by the BCA, i.e., unable to generate new infections (I_b); and vii) removed and colonized by the BCA (R_b). The seven state variables are mutually exclusive so that:

$$K = HS + I + R + H_r + H_b + I_b + R_b \quad (1)$$

The model considers that, during the epidemic and as a consequence of BCA application, the K units move from one state variable to another by means of rates.

At the beginning of a simulation, all of the plant tissue is in the state variable HS. The size of HS is dynamic and increases over time as a consequence of plant growth (in such a way that HS = 1 at the time of maximum plant size), or decreases as a consequence of senescence (which is relevant for those diseases in which the senescent plant tissue is no longer susceptible to infection). Inflow (rate of growth, RG) and outflow (rate of senescence, RS) of host tissue with respect to HS is calculated as follows:

$$RG_t = HS_{t-1} \times RRG_t \quad (2)$$

$$RS_t = HS_{t-1} \times RRS_t \quad (3)$$

in which t is the current day; t-1 is the day before; and RRG_t and RRS_t are relative rates of host growth and senescence on day t, respectively.

The host tissue in the state variable HS moves to state variable I as a consequence of infection by the pathogen; this flow is regulated by RI, the rate of infection, which is calculated as follows:

$$RI_t = STARTP_t + b_t \times I_{t-1} \times COFR_t \quad (4)$$

in which STARTP is the initial inflow of the pathogen into the system; *b* is the relative rate of infection; I is as previously defined; and COFR is the correction factor for occupied tissue.

In equation (4), STARTP is calculated by assuming that the pathogen enters the system starting on day PIN (the day of the first seasonal infection) and continues to enter at a constant rate RPIN for a period of PDUR days; PIN, RPIN, and PDUR are all model parameters that are defined for each situation.

In equation (4), COFR is calculated as follows:

$$COFR_t = (1 - ((K_t - HS_t) / K_t) \quad (5)$$

in which K and HS are as previously defined.

The host tissue in the state variable I moves to state variable R when the infectious period (i.e., the period during which the pathogen continues producing

inoculum on affected tissue) is over; this outflow is regulated by RR, the rate of removal, which is calculated as follows:

$$RR_t = h_t \times I_{t-1} \quad (6)$$

in which *h* is the relative rate of removal, and I is as previously defined.

The model considers that, at any time during the simulation period, a BCA enters the system because of human intervention (i.e., a treatment with the BCA); this can be before, at the same time as, or after the pathogen. The BCA inflow is regulated by STARTB, which is calculated for a period of BDUR days (i.e., the period during which the BCA is applied), starting from day BIN (i.e., the day on which the BCA is applied) at a constant rate equal to RBIN; BIN, RBIN, and BDUR are all model parameters that are defined for each situation.

The introduction of the BCA generates outflows from HS, so that the healthy tissue cannot be infected by the pathogen and, therefore, cannot move to I. The model considers that this outflow can be caused by BCAs that induce resistance in the host tissue and/or that prevent infection due to competition and/or antibiosis.

For BCAs that induce resistance, the outflow from HS (named RRES) is calculated as follows:

$$RRES_t = c_{0\,t} \times H_{r\,t-1} \times COFRH_{r\,t-1} \quad (7)$$

in which c_0 is the relative rate of change from HS to H_r, and $COFRH_r$ is the correction factor for plant resistant tissue and is calculated as follows:

$$COFRH_{r\,t} = (1 - ((K_t - H_{r\,t}) / K_t)) \quad (8)$$

in which K and H_r are as previously described.

For BCAs that prevent infection by the pathogen, the outflow from H_r (named $RCOLH_r$) is calculated as follows:

$$RCOLH_{r\,t} = STARTB_t + c_{1\,t} \times BSUM_{t-1} \times COFRH_{r\,t-1} \quad (9)$$

in which c_1 is the relative rate of change from H_r to H_b; BSUM is the total of the tissue colonized by the BCA (i.e., BSUM= $H_b + I_b + R_b$); and STARTB and $COFRH_r$ are as previously defined.

Because induction of resistance in the host tissue is transitory, the model considers that the H_r tissue can go back to HS and become susceptible to infection. The flow from H_r to HS is calculated as follows:

$$RSUS_t = e_t \times H_{r\,t-1} \quad (10)$$

in which *e* is the relative rate of change from H_r to HS, and H_r is as previously described.

The introduction of a BCA that prevents infection by the pathogen also generates an outflow from HS (named RCOLH), which is calculated as follows:

$$RCOLH_t = STARTB_t + c_{1\,t} \times BSUM_{t-1} \times COFR_{t-1} \quad (11)$$

in which c_1 is the relative rate of change from HS to H_b, and BSUM, STARTB, and COFR are as previously defined.

The introduction of a BCA also generates an outflow from I. This occurs for those BCAs able to inhibit or reduce the sporulation on affected and infectious plant tissue (i.e., I) because of mycoparasitism and/or antibiosis, so that the infectious tissue reduces its ability to generate new infections. The outflow from I (named RCOLI) is calculated as follows:

$$RCOLI_t = STARTB_t + c_{2\,t} \times BSUM_{t-1} \times COFRI_{t-1} \quad (12)$$

in which c_2 is the relative rate of change from I to I_b; BSUM and STARTB are as previously defined; and COFRI is the correction factor for infectious tissue and is calculated as follows:

$$COFRI_t = (1 - ((K_t - I_t) / K_t)) \quad (13)$$

in which K and I are as previously described.

The introduction of a BCA also generates an outflow from R, even though this does not directly affect the epidemic. This outflow (termed RCOLR) is calculated as follows:

$$RCOLR_t = STARTB_t + c_{2\,t} \times BSUM_{t-1} \times COFRR_{t-1} \quad (14)$$

in which c_2 is the relative rate of change from R to R_b; BSUM and STARTB are as previously defined; and COFRR is the correction factor for removed tissue and is calculated as follows:

$$COFRR_t = (1 - ((K_t - R_t) / K_t)) \quad (15)$$

in which K and R are as previously described.

The model considers that as the plant tissue becomes colonized by the BCA (which is accounted for by equations (9), (11), (12), and (14)), the plant tissue can revert to BCA-free tissue because of BCA mortality. The flows from H_b, I_b, and R_b to HS, I, and R, respectively, are calculated through a rate of BCA mortality, BMOR (BMORH, BMORI, and BMORR, respectively), as follows:

$$BMOR_t = f_t \times (H_b \text{ or } I_b \text{ or } R_b)_{t-1} \quad (16)$$

in which f is the relative rate of mortality (i.e., the relative rate of change from H_b, I_b, or R_b to HS, I, or R, respectively).

Driving variables for the pathogen

Driving variables are those functions that determine the relative rate of change of the system as influenced by external variables (Rabbinge and de Wit 1989).

For pathogen infections that are influenced by temperature and relative humidity, the relative rate of infection (*b*) is calculated by equation (17a):

$$b_t = (\gamma \times Teq_t{}^{\zeta} \times (1 - Teq_t))^{\nu} \Big/ \left(1 + exp^{(\varrho - \psi \times \frac{RH_t}{100})}\right) \qquad (17a)$$

in which γ, ζ, and ν are the equation parameters accounting for the effect of temperature; ϱ and ψ are the equation parameters accounting for the effect of humidity; *Teq* are temperature equivalents calculated as (T_t-Tmin)/(Tmax-Tmin), in which T_t is the average temperature (in °C) of day t; Tmin and Tmax are minimal and maximal temperatures at which the pathogen can cause infection, respectively; and RH is the average relative humidity (%) of day t.

For pathogen infections that are influenced by temperature and the duration of a moist period, the relative rate of infection (*b*) is calculated by equation (17b):

$$b_t = (\alpha \times Teq_t{}^{\beta} \times (1 - Teq_t))^{\theta} \times exp^{\,-\vartheta \times exp^{\,(-\varsigma \times MD_t)}} \qquad (17b)$$

in which α, β, and θ are the equation parameters accounting for the effect of temperature; ϑ and ς are the equation parameters accounting for the effect of moisture; *Teq* is as previously described; MD is moisture duration (number of wet hours per day or number of hours with high RH, depending on the pathogen).

Equation (17a) is a logistic equation, and equation (17b) is a Gompertz equation, and both describe the S-shaped increase in infection as "moisture" (RH or MD, respectively) increases (Campbell and Madden 1990) up to an asymptote that is defined by temperature by means of a bell-shaped beta equation of Analytis (Analytis 1977). In the beta equation, parameters γ and α define the top of the curve, ζ and β its symmetry, and ν and θ its size.

The relative rate of change from I to R (*h*) is calculated as follows:

$$h = 1 \Big/ \left\{\phi \times \left[\left(\frac{T_t - Tmin}{Topt - Tmin}\right) \times \left(\frac{Tmax - T_t}{Tmax - Topt}\right)^{\left(\frac{Tmax - Topt}{Topt - Tmin}\right)}\right]\right\} \qquad (18)$$

in which ϕ is the duration of the infectious period (in days) at the optimum temperature (Topt, °C), and T_t, Tmin, and Tmax are as previously described.

In equation (18), the temperature response curve is derived from Reed et al. (1976) and Wadia and Butler (1994).

Driving variables for the BCA

The model considers four main biocontrol mechanisms: mycoparasitism, competition, antibiosis, and induced resistance. As in Jeger et al. (2009), a single BCA can have one or more biocontrol mechanisms, and these may operate additively. The biocontrol mechanisms characterizing an individual BCA are included in the model as the BCA profile (PROF):

$$PROF = P + C + A + IR \quad (19)$$

in which P, C, A, and IR are the relative contribution of mycoparasitism, competition, antibiosis, and induced resistance, respectively, to the overall BCA activity, considering that P + C + A + IR = 1.

The relative rates of change from HS to H_r (c_0), HS and H_r to H_b (c_1), and I to I_b and R to R_b (c_2) are calculated as follows:

$$c_0 = GRO \times IR \times EFF_0 \quad (20)$$

$$c_1 = GRO \times (C + A) \times EFF_1 \quad (21)$$

$$c_2 = GRO \times (A + P) \times EFF_2 \quad (22)$$

in which GRO is the BCA growth rate under fluctuating temperature and moisture; EFF_0, EFF_1, and EFF_2 are overall BCA efficacies in preventing the infection of the HS and H_r tissue by induced resistance (EFF_0), antibiosis, and mycoparasitism (EFF_1), and in reducing the sporulation of the I tissue (EFF_2).

In equations (20), (21), and (22), GRO is calculated by using equation (17b), in which α, β, and θ are replaced by χ, δ, and ε (the equation parameters accounting for the effect of temperature); ϑ and ς are replaced by ω and η (the equation parameters accounting for the effect of moisture); and *Teq* and MD are as previously defined.

The relative rate of change from H_b to HS, I_b to I, and to R_b to R (f) is calculated as follows:

$$f_t = \left\{1 - \left[\left(\frac{T_t - Tmin}{Topt - Tmin}\right) \times \left(\frac{Tmax - T_t}{Tmax - Topt}\right)^{\left(\frac{Tmax - Topt}{Topt - Tmin}\right)}\right]\right\} \times \left\{1 - \left[\left(\frac{RH_t - RHmin}{RHopt - RHmin}\right) \times \left(\frac{RHmax - RH_t}{RHmax - RHopt}\right)^{\left(\frac{RHmax - RHopt}{RHopt - RHmin}\right)}\right]\right\} \quad (23)$$

in which T_t and RH_t are as previously defined, and Tmin, Topt, Tmax, RHmin, RHopt, and RHmax are the minimal, optimal and maximal temperatures and RHs for BCA survival, respectively. The temperature and RH response curve in

polynomial equation (23), in which T and RH are the independent variables, is derived from equation (18).

The relative rate of change from H_r to HS (*e*) is constant and depends on the duration of the induced resistance in the plant tissue, which depends on the combination of BCA and pathogen.

Model output

The model output is represented by changes over time of the state variables in the system. An example of model output is shown in Figure 8.2A for three categories of host tissue: i) healthy and susceptible (HS, green line); ii) healthy and occupied by the BCA (H_b, purple line); and iii) occupied by the pathogen and infectious (I, red line). The simulation describes the changes in the proportion of the three categories of host tissue following the application of a preventative BCA on day 1 for a 40-d period during which the host tissue does not change because of plant growth and/or senescence. In Figure 8.2A, the proportion of HS tissue declines on day 1 because of the introduction of the BCA, which colonizes 60% of the tissue, and declines again at day 4 because of infection by the pathogen. Following infection, the tissue colonized by the BCA remains relatively constant until day 25; during this period, the BCA is effective in controlling the pathogen, which does not colonize additional tissue. After day 25, the tissue colonized by the BCA rapidly decreases, and the tissue occupied by the pathogen increases. Weather conditions (Fig. 8.2B) are important drivers for these dynamics, with a decrease in air temperature and wetness duration favoring the pathogen more than the BCA.

An additional state variable, the area under the disease progress curve (AUDPC) (Campbell and Madden 1990), was calculated to evaluate the overall effects of BCA characteristics and usage and of environmental conditions on the disease development. The AUDPC was calculated at a daily rate (RAUDPC) as the sum of the total K units of plant tissue occupied by the pathogen (POCC) as follows:

$$AUDPC = I + R + I_b + R_b \quad (24)$$

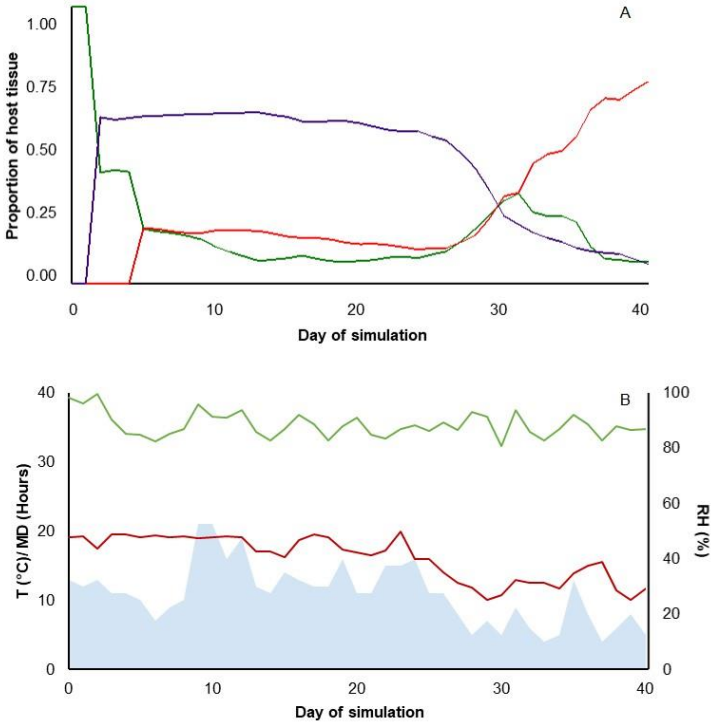

Figure 8.2. A representative simulation of the model, which predicts the control of a foliar pathogen following application of a biocontrol agent. (**A**) Example of one simulation that shows the dynamics for three categories of host tissue: healthy-susceptible tissue (HS, green line), infectious tissue (I, red line), and BCA colonized tissue (H_b, violet line). The simulation refers to the application of a BCA as a preventative treatment for a simulation period of 40 days. (**B**) Weather conditions used as input in this simulation: temperature (T, °C, red line); moisture duration (MD, h, light-blue area); and relative humidity (RH, %, green line).

Model parametrization

The model was parametrized for the biocontrol of BBR in grapevine clusters during ripening, which is between “veraison” (growth stage GS83; Lorenz et al. 2015) and “berries ripe for harvest” (GS89). The simulation period was set at 40 days, with northern Italy as the reference environment (Fedele et al. 2018). K units are single berries, which can dynamically belong to one of the seven categories (state variables) of the model. Because the number of berries is already defined at the beginning of the simulation period (K = 1) and does not change because of plant growth from GS83 to GS89, RRG was set to 0 for the entire simulation. Because we assumed that no berries become resistant to *B. cinerea* infection because of senescence during that growth stage, RRS was set to 0 for the entire simulation.

During ripening, BBR can develop under favorable weather conditions through three main pathways: i) latent infections become visible as rotted berries; ii) air-borne conidia germinate on and infect berries; and iii) aerial mycelium produced on rotted berries infects adjacent healthy berries (berry-to-berry infection) (Elmer and Michailides 2007; González-Domínguez et al. 2015). In the current study, we considered that latent infections and resulting berry-to-berry infections are more common than conidial infections (McClellan and Hewitt 1973; Nair et al. 1995; Pezet et al. 2003; Keller et al. 2003; González-Domínguez et al. 2015). We then assumed that the BBR epidemic starts with the onset of rotted berries that have been latently infected in early growth stages, which constitutes the initial inflow of the pathogen into the system (STARTP). This inflow is assumed to occur on the 4th day of the simulation (i.e., PIN = 4), and to continue for a period of PDUR=1 day, at a rate RPIN=0.2 (meaning that 20% of the berries are affected by latent infections).

During the simulation, new berries become affected (i.e., rotted) through the berry-to-berry pathway at the relative rate *b*, which is calculated by using equation (17a) following Ciliberti et al. (2015b). We assumed that as berries become affected, they begin producing conidia and enter in the I category. Afterwards, the affected berries continue producing conidia until harvest (Elmer and Michailides 2007); therefore, there is no outflow from I to R, and $h = 0$.

Two BCAs with different multiple MOA (i.e., having different PROFs) were entered in the system (i.e., BCAs are applied to clusters) in different simulation runs. Specifically, the MOA profile of the first BCA is PROF = P (0.0) + C (0.8) + A (0.2) + IR (0.0). This profile can represent, for example, *Aureobasidium pullulans*, which is effective against *B. cinerea* by competing for nutrients at the infection site, which is its main MOA, and also by releasing hydrolytic enzymes that inhibit the pathogen (Castoria et al. 2001; Di Francesco et al. 2015) The MOA profile of the second BCA is PROF = P (0.8) + C (0.2) + A (0.0) + IR (0.0). This MOA profile can

represent, for example, to *Pythium oligandrum*, which is mainly a mycoparasite but which also competes for nutrients with pathogens (Lewis et al. 1989).

In the model, the overall BCA efficacies (EFF_0, EFF_1, and EFF_2) in preventing the infection are considered at their maximal (i.e., EFF_0, EFF_1, EFF_2=1), meaning that tissue colonized by the BCA totally prevent or reduce *B. cinerea* development.

Both BCAs are applied to clusters as a preventative treatment on the 1st day of simulation (BIN = 1) or as a curative treatment on the 7th day (BIN = 7). These applications constitute the initial inflow of the BCA into the system (STARTB), which has BDUR=1 day (i.e., the day of BCA application) at a rate RBIN=0.6 (meaning that the BCA covers 60% of the K units at the time of application).

Parameters of driving functions for calculating *b*, GRO, and *f* were derived from the literature and are indicated in Table 8.2. Rate *b* is calculated by using equation (17a) as in Ciliberti et al. (2015b). GRO is calculated by using equation (17b) and by using different parameter values that describe the different responses to temperature and moisture of nine BCA strains (named S1 to S9, see Table 8.2).

Finally, rate *f* is calculated using equation (23) with different settings of parameter values referring to three temperature and humidity conditions under which the BCA survives (Table 8.2); these settings simulate different BCA manufacturing processes and/or formulations that result in different survival capabilities under stressful vineyard conditions (Carbò et al. 2017; Fu and Chen 2011).

Table 8.2. Parameter estimates of the equations fitting the following relationships: the effects of temperature and relative humidity on *b* (the relative rate of *Botrytis cinerea* infection); the effects of temperature and moisture duration on GRO (the relative rate of growth of the BCA); and the effects of temperature and relative humidity on *f* (the relative rate of BCA mortality).

Relative rate		**Parameter**						
b [a]		γ	ζ	ν	ϱ	ψ	Tmin	Tmax
	Botrytis cinerea	7.750	2.140	0.469	35.360	40.260	0	30
GRO [b]	BCA strain	χ	δ	ε	ω	η	Tmin	Tmax
	S1	6.416	1.292	0.469	2.300	0.048	0	35
	S2	12.000	4.000	0.469	2.300	0.048	5	37
	S3	4.000	0.600	0.469	2.300	0.048	0	30
	S4	6.416	1.292	0.469	4.000	0.500	0	35
	S5	12.000	4.000	0.469	4.000	0.500	5	37
	S6	4.000	0.600	0.469	4.000	0.500	0	30
	S7	6.416	1.292	0.469	2.300	0.010	0	35
	S8	12.000	4.000	0.469	2.300	0.010	5	37
	S9	4.000	0.600	0.469	2.300	0.010	0	30
f [c]	Survival capability	Tmin	Tmax	Topt	RHmin	RHmax	RHopt	
	low	0	35	10	0	100	30	
	medium	5	35	15	0	100	40	
	high	5	40	20	0	100	50	

[a] $b = (\gamma \times Teq^{\zeta} \times (1 - Teq))^{\nu} / (1 + exp^{(\varrho - \psi \times RH/100)})$; *b* is the relative infectious rate; *Teq* is the equivalent of temperature calculated as $(T_t\text{-Tmin})/(\text{Tmax-Tmin})$, in which T is the average temperature (in °C), and RH is the average relative humidity (%).

[b] $\text{GRO} = (\chi \times Teq\delta \times (1 - Teq))^{\varepsilon} \times exp^{-\omega \times exp^{(-\eta \times MD)}}$; GRO is the BCA growth rate under *Teq* as previously described; *MD* is moisture duration.

[c] $f = \{1\text{-}[((T - \text{Tmin}) / (\text{Topt} - \text{Tmin})) \times ((\text{Tmax} - T) / (\text{Tmax} - \text{Topt}))^{((\text{Tmax} - \text{Topt}) / (\text{Topt} - \text{Tmin}))}]\} \times \{1 - [((\text{RH} - \text{RHmin}) / (\text{RHopt} - \text{RHmin})) \times ((\text{RHmax} - \text{RH}) / (\text{RHmax} - \text{RHopt}))^{((\text{RHmax} - \text{RHopt}) / (\text{RHopt} - \text{RHmin}))}]\}$; T and RH are as previously defined; Tmin, Topt, Tmax, RHmin, RHopt, and RHmax are minimal, optimal, and maximal temperatures and relative humidity, respectively, for BCA survival.

Model running

The model was used to study the effect of the following sources of variation on BBR development: i) MOA of the BCA (2 levels: mainly competition and mainly mycoparasitism); ii) BCA application time (2 levels: preventative and curative); iii) BCA strain (9 levels: 3 ranges of temperatures combined with 3 moisture requirements for BCA growth); and iv) BCA survival capability (3 levels: low, medium, and high). These sources of variation generate 108 combinations (2 MOAs x 2 application times x 9 strains x 3 survival capabilities). In addition, a situation with no BCA application was considered as the untreated control (NT). To study the effect of environmental conditions, each combination was run under nine scenarios that reflect three climate types with three scenarios per type: i) warm and dry; ii) mild and semi-arid; and iii) cold and wet. Scenarios are represented by fluctuating conditions of temperature, relative humidity, and wetness duration (see Table 8.3). Therefore, 981 model runs were generated: (1 NT + 108 BCA combinations) x 9 climate scenarios.

An example of the effect of the previously mentioned sources of variation on the disease dynamics in the three climate types is provided in Figure 8.3. Each graph shows the simulated proportion of the host tissue occupied by the pathogen (POCC) over the entire simulation period for each climate type (blue lines, cold and wet; green lines, mild and semi-arid; and yellow lines, warm and dry; Fig. 8.3). Simulations of Figure 8.3 refer to a competitive BCA (Fig. 8.3A and 8.3B) or to a mycoparasitic BCA (Fig. 8.3C and 8.3D) applied as a preventative treatment (Fig. 8.3A and 8.3C) or as a curative treatment (Fig. 8.3B and 8.3D), with the temperature and moisture requirements of S8, and with low survival capability (Table 8.2). In the cold and wet climate type, BCA application reduced the final (i.e., at day 40) value of POCC by 11 to 16%. In the mild and semi-arid climate type, BCA application reduced the final value of POCC by 53 to 68%, irrespective of application time or MOA. In the warm and dry climate type, BCA application reduced the final value of POCC by 40 to 45%.

The final values of AUDPC simulated for each of the 981 runs of the model were used to calculate the efficacy (E) of each BCA combination (T) in relation to the untreated control (NT) as follows: $E = (NT - T) / NT$. A factorial analysis of variance (ANOVA) was carried out for each climate type to determine whether the efficacy of each BCA combination was signicantly affected by the main sources of variation (MOA, application time, strain, survival capability) or their interactions. The three scenarios per climate type were used as replicates. The ANOVA was conducted by using the function *anova* of R software (v 3.6.0; R core team, 2019).

Figure 8.4 summarizes the efficacy of the BCA in controlling BBR for the different simulation runs. Under the cold and wet climate (Fig. 8.4A), in which BBR

developed rapidly and occupied all of the host tissue in < 20 days (Fig. 8.3), BCA efficacy ranged from 0 to 99% and was significantly influenced by all of the main sources of variation (P<0.001) and by the following interactions: MOA × application time, application time × strain, application × survival capability, and strain × survival capability (Table 8.4). MOA and application time (preventative or curative) accounted for 2.6% and 1.7% of total variance, respectively, and all of the interactions accounted for <1.7% of the total variance (Table 8.4). Those factors that are greatly affected by environmental conditions (the BCA strain and its survival capability) together accounted for 91% of the total variance (Table 8.4). The average efficacy was higher for BCAs with medium or high survival capability (Fig. 8.5A) than for BCAs with low survival capability. The average efficacy in the cold and wet climate was higher for S2 and S8 than for S6 (Fig. 8.6).

Under the mild and semi-arid climate (Fig. 8.4B), in which BBR developed gradually and did not completely occupy the host tissue until the end of the simulation period (Fig. 8.3), BCA efficacy ranged from 1 to 71% and was significantly influenced by the MOA of the BCA (P = 0.017), which accounted for 0.8% of total variance (Table 8.4), and by the growth requirement of the BCA (as indicated by the strain, P = 0.002), which accounted for 0.4% of total variance (Table 8.4). BCA efficacy was significantly affected (P<0.001) by the survival capability of the BCA, which accounted for 97.9% of the total variance (Table 8.4); BCA efficacy increased with the survival capability of the BCA (Fig. 8.5B).

Under the warm and dry climate (Fig. 8.4C), in which BBR developed slowly and occupied only 50% of the host tissue at the end of the simulation period (Fig. 8.3), BCA efficacy ranged from 0 to 40% and was significantly influenced by the survival capability of the BCA (P<0.001) and by the interaction between application time and survival capability (P=0.007), which accounted for 97.3 and 2.2% of total variance, respectively (Table 8.4). The average efficacy was higher for BCAs with a high survival capability than for BCAs with a low or medium survival capability (Fig. 8.5C). MOA, strain, and application time did not significantly affect BCA efficacy (Table 8.4).

Table 8.3. Summary of the weather data for the nine climate scenarios.

Climate type	Scenario	Average temperature (°C) [a]	Average relative humidity (%) [b]	Total wetness duration (h) [c]
Warm and dry	1	25.74	68.80	56
	2	25.40	74.85	70
	3	25.88	69.75	61
Mild and semi-arid	1	19.15	79.27	81
	2	20.08	79.35	91
	3	18.74	80.97	112
Cold and wet	1	17.08	87.32	588
	2	15.37	87.55	287
	3	16.16	88.30	446

[a] Average of daily temperatures (°C).
[b] Average of daily relative humidity (%).
[c] Total number of hours with wetness (h).

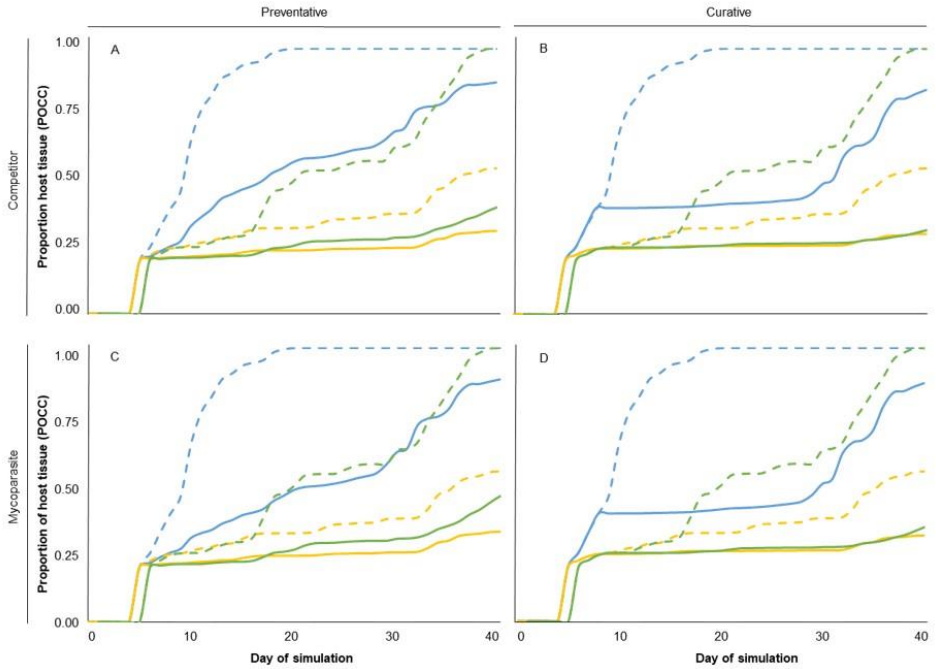

Figure 8.3. Examples of the total tissue occupied by the pathogen (POCC) as affected by MOA, application time, and climate type. **A**) A mainly competitive BCA applied as a preventative treatment; **B**) A mainly competitive BCA applied as a curative treatment; **C**) A mainly mycoparasitic BCA applied as a preventative treatment; **D**) a mainly mycoparasitic BCA applied as a curative treatment. Dashed lines indicate POCC dynamics when no BCA application is applied (NT), and solid lines indicate POCC dynamics when a BCA is applied. Blue, green, and yellow lines indicate the simulation in a cold and wet, mild and semi-arid, and warm and dry climate type, respectively. Each line corresponds to the POCC dynamics averaged across the three scenarios used as replicates for each climate type.

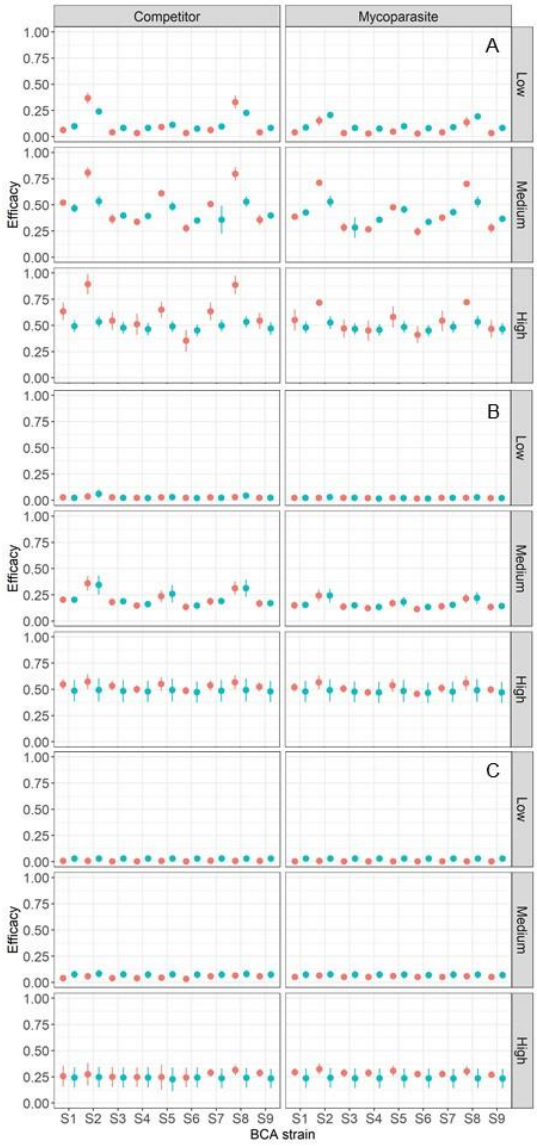

Figure 8.4. BCA efficacy against Botrytis bunch rot in ripening grapevine clusters as affected by MOA (mainly competitor or mainly mycoparasite, as indicated at the top of the figure), responses of 9 BCA strains to temperature (S1 to S9; X axis, see Table 8.2 for details), BCA survival capability (low, medium, and high, as indicated on the right side of each plot), and climate(**A**: cold and wet; **B**: mild and semi-arid; **C**: warm and dry). Each point represents the average, and the bars represent the standard errors of three scenarios per each climate type. Red and blue colors indicate that the BCA is applied as a preventative or a curative, respectively, i.e., before or after *Botrytis cinerea* infection.

Table 8.4. Analysis of variance statistics for the influence of MOA, application time, BCA strain, and survival capability on BCA efficacy.

		Warm and dry [a]		Mild and semi-arid		Cold and wet	
Source of variation	**df [b]**	**Variance [c]**	**P(>F)**	**Variance**	**P(>F)**	**Variance**	**P(>F)**
MOA	1	0.05	0.724	0.763	0.017	2.59	<0.001
Application time	1	0.03	0.791	0.155	0.279	1.66	<0.001
Strain	8	0.03	1.000	0.412	0.002	4.96	<0.001
Survival capability	2	97.34	<0.001	97.884	< 0.001	86.02	<0.001
MOA × Application time	1	0.14	0.562	0.009	0.788	1.31	0.001
MOA × Strain	8	0.02	1.000	0.015	0.998	0.10	0.553
MOA × Survival capability	2	0.05	0.879	0.264	0.137	0.06	0.626
Application time × Strain	8	0.03	1.000	0.011	1.000	1.04	<0.001
Application time × Survival capability	2	2.15	0.007	0.311	0.097	1.68	<0.001
Strain × Survival capability	16	0.01	1.000	0.137	0.417	0.25	0.010
MOA × Application time × Strain	8	0.02	1.000	0.002	1.000	0.10	0.547
MOA × Strain × Survival capability	16	0.01	1.000	0.017	1.000	0.04	0.991
MOA × Application time × Survival capability	2	0.08	0.823	0.008	0.949	0.03	0.799
Application time × Strain × Survival capability	16	0.01	1.000	0.012	1.000	0.12	0.446
MOA × Application time × Strain × Survival capability	16	0.01	1.000	0.000	1.000	0.03	0.999

[a] To study the effect of environmental conditions, each combination was run under nine contrasting scenarios, i.e., under three scenarios for each of three climate types.
[b] Degrees of freedom.
[c] Percentage of the variance accounted for by each source of variation.

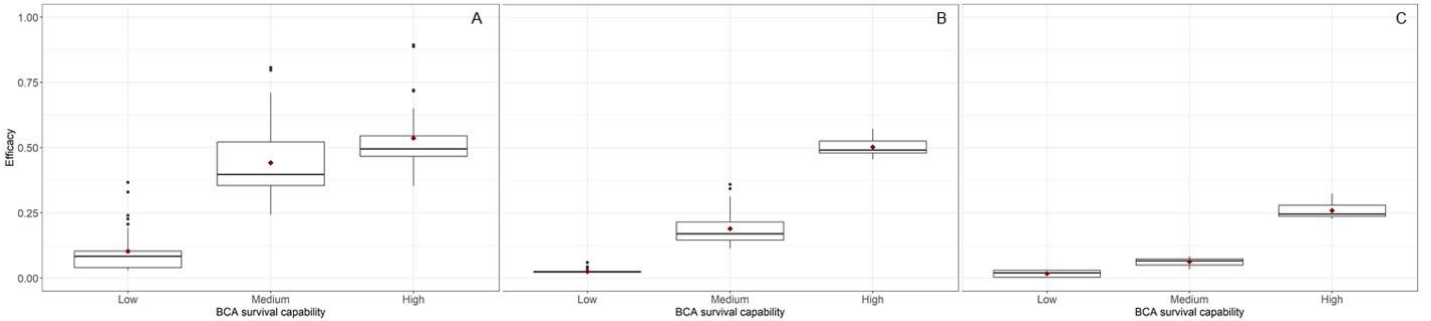

Figure 8.5. BCA efficacy against Botrytis bunch rot in ripening grapevine clusters averaged across nine BCA strains and as affected by BCA survival capability (low, medium, and high; see Table 8.2) and climate type (**A**: cold and wet; **B**: mild and semi-arid; **C**: warm and dry). The thick line in the boxes is the median; the lowest value in each box represents the 1st quartile (25th percentile); the highest value of each box represents the 3rd quartile (75th percentile); red points in the boxes are the means; black points in the graph are outliers.

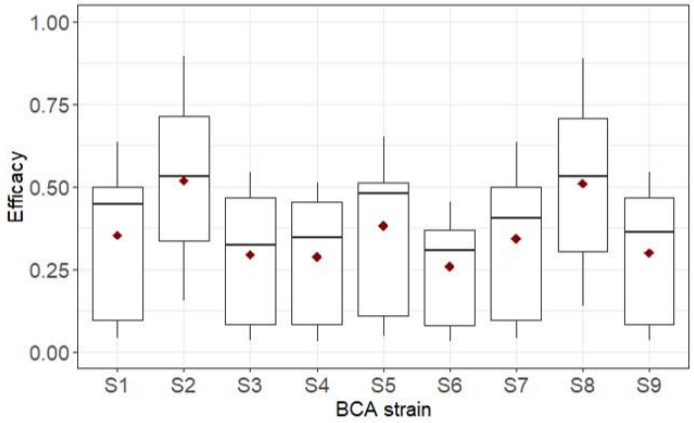

Figure 8.6. BCA efficacy against Botrytis bunch rot in ripening grapevine clusters of nine BCA strains (see Table 8.2) in the cold and wet climate. The thick line in the boxes is the median; the lowest value in each box represents the 1st quartile (25th percentile); the highest value of each box represents the 3rd quartile (75th percentile); red points in the boxes are the means; black points in the graph are outliers.

Discussion and conclusion

The model that was developed by Jeger et al. (2009) and that was improved by Xu et al. (2010), Xu et al. (2011), and Xu and Jeger (2013) accounts for the biocontrol mechanisms involved and is able to predict the dynamics of pathogen and biocontrol agent (BCA) populations. In Xu and Jeger (2013), the significant effects of varying BCA–temperature relationships and application times on BCA efficacy suggested the importance of considering environmental conditions under which the BCA and target pathogen interact. In the present research, the model of Jeger et al. (2009) was enlarged to include crop growth and senescence, and the environmental effects on the pathogen and on BCA growth and survival. Like the model of Jeger et al. (2009), the enlarged model has a generic structure and can be applied to any pathosystem involving fungal pathogens of aerial plant organs as well as different pathogen–BCA interactions involving different BCA mechanisms of action.

We parametrized the enlarged model for *B. cinerea* causing Botrytis bunch rot (BBR) on grapevines. The model parametrization was derived from the epidemiological studies performed by Ciliberti and colleagues (Ciliberti et al. 2015a; 2015b; 2016). Those epidemiological relationships were incorporated in a mechanistic model for *B. cinerea*-grapevine developed by González-Domínguez et al. (2015), but the latter model did not include a BCA component. The use of BCAs for BBR control has been extensively studied (Abbey et al. 2018; Elad and

Stewart 2007; Elmer and Reglinski 2006; Sharma et al. 2009; Jacometti et al. 2010; Haidar et al. 2016), with emphasis on biocontrol mechanisms and field efficacy; less research has been conducted to understand how environmental conditions affect BCA fitness and efficacy (Fedele et al. 2019a, see Chapter 7). In the current study, the model parametrization for BCAs used different parameter values represented by nine BCA strains, which differed in their growth and survival in response to temperature and moisture conditions.

The model was run under three climate types to study the combined effects of the following factors: (i) mechanism of action of the BCA; (ii) timing of BCA application with respect to the pathogen (preventative vs. curative); (iii) temperature and moisture requirements for BCA growth; and iv) BCA survival capability. All of these factors affected, although to different degrees, biocontrol efficacy. Environmental conditions were the most important factors, accounting for >90% of the variance in simulated biocontrol efficacy; other factors, even though significant under some climate types, accounted for only a minor percentage of the variance. This finding may help explain why the application of BCAs often results in inconsistent control of the target pathogen in the field (Haidar et al. 2016). In other words, our results suggest that the inconsistent BCA efficacy in repeated experiments (Guetsky et al. 2001; Hannusch and Boland 1996; Shtienberg and Elad 1997) and in the practical biocontrol of diseases (Elmer and Reglinski 2006; Fravel 1999; Huang et al. 2000; Stewart 2001) can be caused, at least to some extent, by differences in environmental conditions between experiments or by fluctuations in environmental conditions in the same experiment (Elad and Freeman 2002; Kredics et al. 2003; Xu et al. 2010). This finding also stresses the importance of considering the environmental response of the BCA during its selection, BCA survival capability during both selection and formulation, and weather conditions and forecasts at the time of BCA application in the field.

Concerning the environmental response of the BCA during its selection, BCAs that are able to grow under a wide range of environmental conditions (i.e., strains S2 and S8 in this study) and that share the temperature and moisture requirements of the target pathogen may be more effective than BCAs with a more limited ability to grow under a range of environmental conditions. BCA response to temperature and moisture can be evaluated by means of environmentally controlled experiments (Fedele et al. 2019b), and the effects of temperature and moisture on the pathogen–BCA relationship can be evaluated by using environmental niches (Fedele et al. 2019a, see Chapter 7). It is essential that the effects of environment be included when screening BCAs for market development (Calvo-Garrido et al. 2018; Köhl et al. 2011).

Concerning the BCA survival capability during both selection and formulations, our model simulations indicate that BCAs may be more effective in controlling the target pathogen for long periods and under a range of weather conditions if they have a high rather than a low survival capability. This result confirms previous findings (Magan 2006; Calvo-Garrido et al. 2014a; Longa et al. 2008) and also the importance of protective effects provided by additives or adjuvants used in the formulation of the commercial product (Calvo-Garrido et al. 2014b; Carbó et al. 2017; Lahlali and Jijakli 2009). This result also confirms that survival capability should be a key property used to screen microorganisms for biocontrol (Köhl et al. 2011).

Finally, weather conditions and forecasts at the time of BCA application in the field should be considered so as to maximize the probability that the BCA will grow and control the pathogen. Although the current model could be useful in this respect, its utility should be verified with field experiments (Gent et al. 2013). On the other hand, developers of BCAs could use the current model to predict the efficacy of candidate organisms under different scenarios of weather conditions and application timings.

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Chapter 9

Chapter 9

CONCLUSIONS

This Thesis covered a wide study on the epidemiology and management of *Botrytis cinerea* affecting grapevines, causing Botrytis bunch rot (BBR). A previous work (Ciliberti 2014) increased our knowledge about the biology and epidemiology of *B. cinerea* and developed a mechanistic, weather driven model to predict the severity of BBR at harvest. Results of this work questioned the current management strategy of BBR, which is based on empiric application of fungicides at four vine growth stages: flowering (A), pre-bunch closure (B), veraison (C), and pre-harvest (D). In some viticultural areas, spraying at timing A has been considered much less effective than spraying in B (Corvi and Tullio 1979; Pérez-Marín 1998); as a consequence, spraying in B rather than in A is recommended. This Thesis goes through some aspects related to this strategy to propose a new approach in BBR management, based on robust scientific knowledge rather than on empiricism.

In **Chapter 2**, a network meta-analysis was used to integrate the results of different strategies (based on combinations of 1, 2, 3, or 4 sprays applied in A, B, C, and/or D) for BBR control. This multi-treatment analysis can also use a large number of individual studies, because it does not require that all of the studies include all of the treatments to be compared; in this case, results from 116 studies conducted between 1963 and 2016 in nine countries were used. Practical recommendations for BBR control should be based on the findings of this study: i) spraying in A seems to be very useful for achieving efficient and flexible BBR control in vineyards; ii) spraying in B instead of A does not provide the same flexibility because, if the grower initially decides to adopt a 1-spray strategy and the season subsequently becomes highly favorable for *B. cinerea*, the grower would no longer be able to adopt strategy ABCD; iii) the BC or BCD strategies, which are still possible if a spray is not applied in A, provide the same control as AC or less control than ACD; strategy BD provides good average control, but comparison with AD was not possible because the latter strategy was not evaluated in this work; and iv) spraying in B is useful only if the grower decides to adopt the ABCD strategy; otherwise, AC or ACD provide satisfactory solutions for 2- or 3-spray strategies, respectively.

In **Chapter 3**, the interactions among fungicide treatments applied at different timings for the control of BBR in vineyards were evaluated, using the same dataset used in Chapter 2. The work focused on strategies in which early- (i.e., in A and B) and late- (i.e., in C and D) season applications are combined. Early-season sprays showed non-additive interactions (i.e., the observed efficacy was significantly lower than expected in case of additive effect) while late-season

sprays did. No significant synergistic effects were observed among fungicide sprays. These results show that the subsequent application of fungicides controlling the same pathway is not fully beneficial in the early-season. These findings support the results obtained in Chapter 2, which outline that spraying in A is more convenient than in B, and performing both sprays is only recommended under conditions of high BBR pressure, in which the strategy ABCD is recommended. Otherwise, strategies CD or ACD are the most convenient, being able to exploit the additive effect of the interventions on repeated infection events (for CD) or different infection pathways of *B. cinerea* (for ACD).

In **Chapter 4**, the effect of early-season fungicide treatments was investigated. For this purpose, a hydrolysis probe-based qPCR assay was optimized for the quantification of *B. cinerea* DNA in grape bunch trash and the calculation of a colonization coefficient (CC), as a tool for investigating the relationships between the quantity of *B. cinerea* DNA, the colonization of, and the sporulation potential on bunch trash. Results indicate that the qPCR assay and CC calculation provide a sensitive and reliable method for quantifying colonization by *B. cinerea* of the trash materials remaining in grape bunches after flowering. The qPCR assay provides consistent results with the traditional methods used to estimate *B. cinerea* colonization of and sporulation on bunch trash. The validity of the qPCR assay was confirmed in the field using bunch trash naturally colonized with *B. cinerea* that had been treated or not treated with fungicides during flowering. In the field, the CC values were consistent with the reduction of the sporulation potential caused by fungicide treatment and revealed differences between fungicide-treated and non-treated plants under different environmental conditions, even in situations where the incidence of colonization of bunch trash by *B. cinerea* was very low. The CC values of bunch trash were also consistent with the reduction in the incidence of latent infection of berries caused by fungicide application at flowering.

In **Chapter 5**, the effects of fungicides (FUN, a commercial mixture of fludioxonil and cyprodonil), biological control agents (BCA, *Aureobasidium pullulans* and *Trichoderma atroviride*), and botanicals (BOT, a commercial mixture of eugenol, geraniol, and thymol) applied at different timings (A, B, C, or ABC) on *B. cinerea* bunch trash colonization and sporulation in vineyards were investigated. The ability of *B. cinerea* to colonize the bunch trash (as indicated by *B. cinerea* DNA content obtained using the qPCR assay optimized in Chapter 4) and to sporulate on bunch trash (as indicated by the number of conidia produced under optimal laboratory conditions) was highly variable, and this variability was higher between years (2015 to 2018) than among the vineyards and the sampling times (i.e., 1 week after applications at A, B, and C). *Botrytis cinerea* sporulation on

bunch trash was significantly lower in plots treated with FUN than in non-treated in only 3 of 18 cases (3 vineyards × 2 years × 3 sampling times). The probability of the applications being effective increased when the sporulation potential on the non-treated bunch trash increased (i.e., when the bunch trash colonization increased), supporting the positive relationship between sporulation potential and CC found in Chapter 4. FUN applications significantly reduced *B. cinerea* colonization of bunch trash compared to non-treated, BCA efficacy was similar to that of FUN, but BOT efficacy was variable. For all products, colonization reduction was the same with application at A vs. ABC, meaning that the effect of an early season application lasted from flowering to one week after veraison. These results confirm the important role of the early-season control of *B. cinerea* (Chapters 2 and 3) in reducing the saprophytic colonization of bunch trash and the subsequent sporulation, especially when the risk of BBR is high.

The estimation of the risk of colonization during the early-season would help growers decide whether an early spray application would reduce the sporulation potential later in the season. This decision, together with that of how many sprays are necessary to control BBR, can clearly be made easier by use of a mathematical model that is able to predict the risk of the disease development. A recently published mechanistic model for *B. cinerea* predicts (González-Domínguez et al. 2015), on a daily basis, the relative infection severity during two infection windows corresponding to the two grape-growing periods relevant for *B. cinerea* infection: i) between “inflorescences clearly visible” and “berries groat-sized, bunches begin to hang”; and ii) ripening berries. The model, which is based on relative infection severity values, predicts the final BBR as mild, intermediate, or severe.

In **Chapter 6**, the model developed by González-Domínguez et al. (2015) was further validated in 23 independent Botrytis bunch rot (BBR) epidemics (combinations of vineyards × year) occurred between 1997 and 2018 in Italy, France, and Spain. The ability of the model to account for latent infections was studied performing shelf-life assays with mature berries with no rot BBR signs. The model correctly classified the severity of 15 of 23 epidemics (65% of epidemics) when the classification was based on field assessments of BBR severity; when the model was operated by considering the BBR severity after shelf-life, its ability of correctly predict the epidemic severity increased from 65% to >87%. This result showed that the model correctly accounts for latent infections. This is an important aspect, considering the relevant role of latent infections occurring from grapevine flowering and onwards in the disease epidemiology. Therefore, the model may be considered a reliable tool for supporting decision making for BBR control in vineyards.

Once a disease control intervention is decided, a further decision is needed, concerning the use of fungicides, botanical or biocontrol agents. In Chapter 4, BCAs were successfully used for early-season applications.

In **Chapter 7**, a systematic literature review was conducted to retrieve and analyze the metadata on the influence of environmental conditions on BCA fitness and efficacy against *B. cinerea*. The review considered 54 papers (selected from a total of 347 papers) and 27 genera of BCAs. The review showed that only limited information is available about the effects of temperature, humidity, and pH on BCA fitness and efficacy. Metadata were also used to define environmental niches for *B. cinerea* and for two BCAs, *Trichoderma* and *Candida*, which were used as case studies. The environmental niches, in turn, were used to study the temperature and humidity conditions under which the BCA prevails over *B. cinerea*, and to define the extent of environmental niche sharing between the BCA and the target pathogen. The concept of environmental niches proposed in Chapter 7 may help researchers identify those BCAs that occupy (or partially occupy) the same niche as the target pathogen; such BCA may therefore have an increased probability of growing under the same environmental conditions under which *B. cinerea* grows; this may lead to greater interaction (which can be parasitism, competition or antagonism, depending on the BCAs' characteristics) between the microorganisms and therefore to higher efficacy of the BCA.

In **Chapter 8**, these considerations were used for the development of a model addressing the effect of environment on BCA-plant pathogen systems for the prediction of BCA effectiveness. A model previously developed by Jeger et al. (2009), was integrated with new components to consider the effect of environmental conditions on the pathogen-BCA interactions and the dynamics of the host, including crop growth and senescence. The model was parametrized for *B. cinerea* causing BBR on grapevines. The effects of the following factors on the BBR development were studied under three climate types through the model: (i) mechanism of action of the BCA; (ii) timing of BCA application with respect to the pathogen; (iii) temperature and moisture requirements for the BCA to grow; and iv) BCA survival capability. All these factors affected the biocontrol efficacy. The two factors affected by environmental conditions (iii and iv) accounted for >90% of the experimental variance, resulting the most important influencing factors for BCA efficacy. These findings stress the importance of considering three main aspects for the selection of new BCAs and their practical use: i) the environmental response of the BCA during its selection; ii) the BCA survival capability during both selection and formulation; and iii) weather conditions and forecasts at the time of BCA application in the field.

Overall, the results provide new information and data for reasonable scheduling of fungicide applications and for an appropriate selection of control products to manage BBR in vineyards.

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