Reduction of *Botrytis cinerea* Colonization of and Sporulation on Bunch Trash

Giorgia Fedele,[1] Elisa González-Domínguez,[2] Melissa Si Ammour,[1] Luca Languasco,[1] and Vittorio Rossi[1,†]

[1] Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy
[2] Horta srl, 29122 Piacenza, Italy

Abstract

Botrytis bunch rot (BBR) of grapevine, caused by *Botrytis cinerea*, is commonly managed by fungicide (FUN) sprays at flowering (A), at pre-bunch closure (B), at veraison (C), and before harvest. 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*. This study investigated the effects of FUNs (a commercial mixture of fludioxonil and cyprodonil), biological control agents (BCAs; *Aureobasium pullulans* and *Trichoderma atroviride*), and botanicals (BOTs; a commercial mixture of eugenol, geraniol, and thymol) applied at different timings (A, B, C, or ABC) compared with a nontreated 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 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). *B. 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 with 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 versus 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.

Keywords: biocontrol, botanicals, bunch trash disinfestation, grey mold, *Vitis vinifera*

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). *B. 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 (GSs) (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, and then conidial germination and extensive colonization of floral debris in grape bunches (pathway III) occurs (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 GSs: end of flowering (A; GS69) (Lorenz et al. 1995), pre-bunch closure (B; GS77), veraison (C; GS83), and before harvest (D; 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 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 (Keller et al. 2003; McClellan and Hewitt 1973; Seyb et al. 2000).

The Directive 128/2009/EC on the Sustainable Use of Pesticides makes it mandatory for the European Union 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 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 FUNs for the control of BBR in vineyards (Calvo-Garrido et al. 2014b, 2019; 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

†Corresponding author: V. Rossi; vittorio.rossi@unicatt.it

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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 FUNs (a commercial mixture of fludioxonil and cyprodonil), biological control agents (BCAs; *Aureobasium pullulans* and *Trichoderma atroviride*), and botanicals (BOTs; a commercial mixture of eugenol, geraniol, and thymol) for bunch trash disinfestation in vineyards. Two experiments were conducted with the following objectives: i) evaluate the effect of different timings of FUN 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

Experiment 1: 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 Castell'Arquato (CA) vineyard is located at CA (44°51′26.1″ N, 9°51′20.7″ E, 400 m above sea level [a.s.l.]) in the Emilia-Romagna region. The Mandriole (MA) vineyard is located at MA (44°41′57″ N, 12°19′66″ E, 0 m a.s.l.), which is also in the Emilia-Romagna region; and the Cormons (CO) vineyard is located at CO (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 cultivar Merlot, and the MA vineyard was planted with cultivar 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 they were trained using a Guyot system; the within- and between-row spacings were 1.0 and 2.3 m, respectively. The vines in the MA vineyard were 11 years old in 2015, and they were trained using the Casarsa system; the within- and between-row spacings were 1.0 and 3.0 m, respectively. The vines in the CO vineyard were 6 years old in 2015, and they were trained using the Guyot system; the within- and between-row spacings were 0.8 and 2.4 m, respectively. Powdery and downy mildews were controlled according to an integrated pest management 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, RH, wetness duration, and rainfall were recorded by an automated weather station (iMeteos; Pessl Instruments GmbH) located <1 km from the experimental plot. GSs of vines 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 (prebunch closure; GS77), C (veraison, GS83), or ABC. A nontreated 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.) at the label dose (800 g/ha) was applied until runoff using a 15-liter Elettroplus knapsack sprayer (Davide e Luigi Volpi S.p.A.). Dates of application are indicated in Figure 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 to 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% RH, and they 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 it 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 gram of dry weight.

Experiment 2: 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); an 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.) 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.) at the label dose (4,000 ml/ha); and iii) BCA, *Aureobasidium pullulans* (Botector; Manica S.p.A.) only at A and C and *Trichoderma atroviride* (Vintec; Belchim Crop Protection Italia S.p.A.) only at B (Pertot et al. 2017); the BCA products were sprayed at the label doses (400 and 1,000 g/ha, respectively). All products were applied until runoff using a 15-liter Elettroplus knapsack sprayer (Davide e Luigi Volpi S.p.A.). Dates of application are indicated in Figure 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 to 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) 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- to 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 EDTA, 1.4 M NaCl, and 1% polyvinylpyrrolidone). The tubes were placed in a Mixer Mill MM200 (Retsch GmbH) for 1 min at 30 cycles per second. 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; vol/vol) was added. After further vigorous mixing, the tubes were centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred to a new microcentrifuge tube. The chloroform-isoamyl alcohol purification was 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 (vol/vol). A third chloroform-isoamyl alcohol purification and centrifugation process was performed, and the resulting supernatant was transferred to a new microcentrifuge tube, to which an equal volume of cold (approximately 0°C) isopropanol and a 10% volume of 3 M sodium acetate was added; this was followed by centrifugation at 12,000 rpm for 5 min at 4°C. The pellet was washed with 70% (vol/vol) ethanol, air dried, and resuspended in 100 μl of sterile distilled water. The yield and purity of the extracted DNA were determined using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific Inc.). The extracts were adjusted to 20 ng/μl of DNA.

Real-time PCR. A duplex quantitative PCR (qPCR) assay was used to assess the colonization of bunch trash by *B. cinerea* as described by Si Ammour et al. (2019). The primers/hydrolysis probe set Bc3 was used to amplify the intergenic spacer region (IGS) region 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 1× QuantiTect Multiplex PCR Kit, 150 nM *V. vinifera* probe ResP, 150 nM *B. cinerea* probe Bc3P, 100 nM each *V. vinifera* primer (Res F/R), 500 nM 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.) with an initial incubation at 95°C for 15 min followed by 40 cycles of

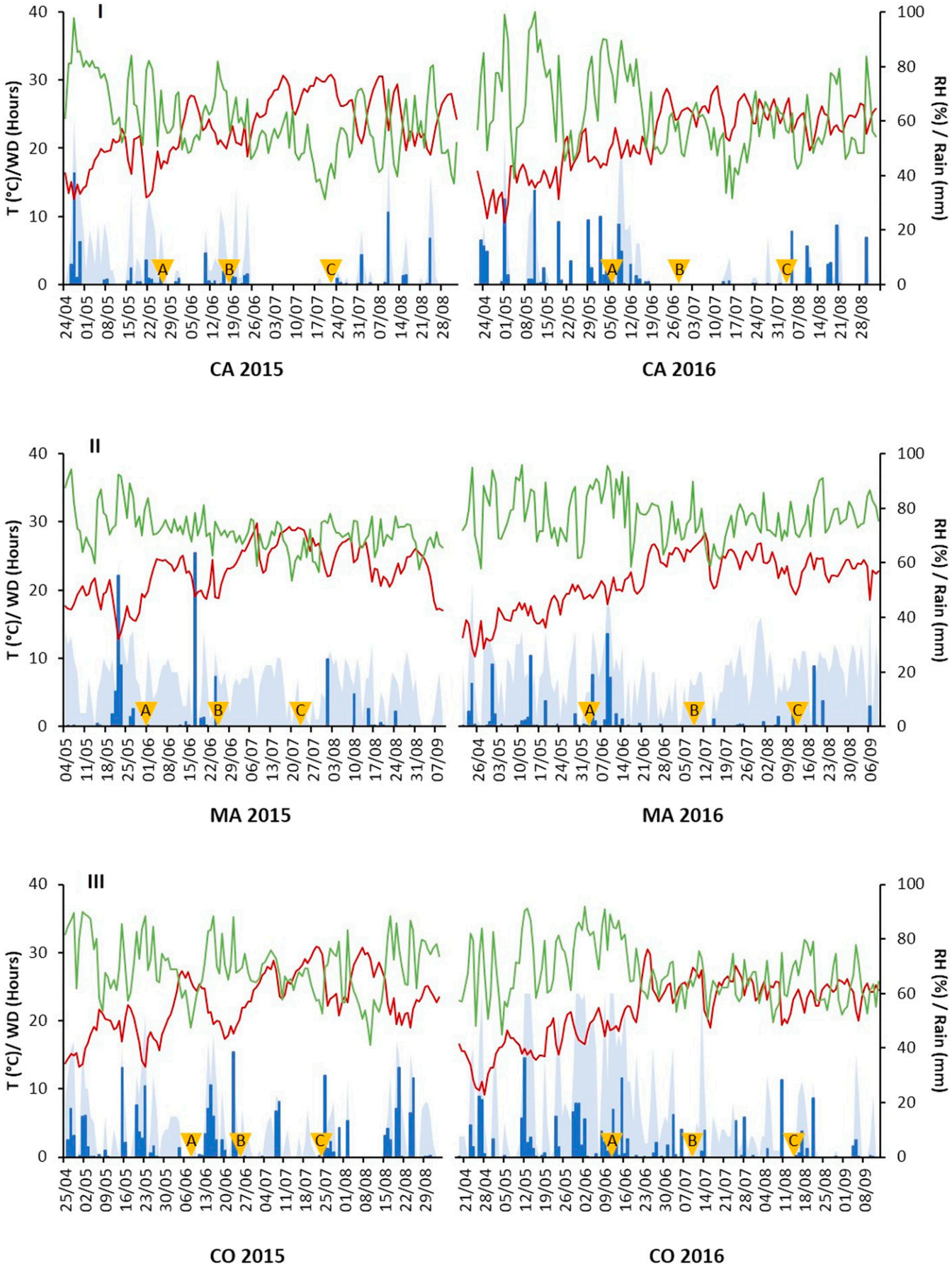

Fig. 1. Weather conditions in the Castell'Arquato (CA), Mandriole (MA), and Cormons (CO) vineyards in 2015 and 2016 (experiment 1). Daily values of temperature (T; red line; in degrees Celsius), RH (green line; in percentage), rain (blue bars; in millimeters), and wetness duration (WD; light blue area; 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).

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 nanograms per microliter) were obtained by transforming the quantification cycles 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). 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).

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 per gram of bunch trash assessed in three vineyards (CA, MA, and CO) in 2 years (2015 and 2016) in plots treated with FUN and NT at three sampling times (1 week after FUN application at A, B, and C). The dataset analyzed for experiment 2 consisted of the CC of *B. cinerea* in bunch trash assessed in the CA vineyard in 2 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 seventh day after veraison (i.e., 1 week after application at C).

In a preliminary analysis conducted with the nonparametric Kruskal–Wallis test (by using the function kruskal.test), the numbers of conidia (experiment 1) and the CC (experiment 2) in the nontreated bunch trash strongly and significantly ($P < 0.001$) differed between the 2 years of each experiment. Therefore, the data were analyzed separately for the 2 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 1) or in an interaction with vineyard (models 2, 4, 6, 8, 10, and 12 in Table 1). In these models, the log link function (transformation) was used for the number of *B. cinerea* conidia per gram of bunch trash, and the quasi-Poisson distribution of errors was selected to compensate for overdispersion (Crawley 2013) owing 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 alone (models 1 and 4 in Table 2), fixed factors with the inclusion of timing of applications (models 2 and 5 in Table 2), or an interaction with timing of applications (models 3 and 6 in Table 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 of more than two 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 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. Afterward, 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 nontreated 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 another analysis, the outcome of FUN application in experiment 1 was considered as zero (i.e., no significant reduction of sporulation compared with NT) or one (i.e., significant reduction). The relationship between this outcome and the number of conidia per gram 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, respectively. To evaluate the effect of the sporulation level on the significant reduction after a FUN application, a chi.test of this model was conducted by using the function anova.

Results

Sporulation of *B. cinerea* on bunch trash as affected by the timing of fungicide application (experiment 1). Weather conditions at the three vineyards and in the 2 years were different (Fig. 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

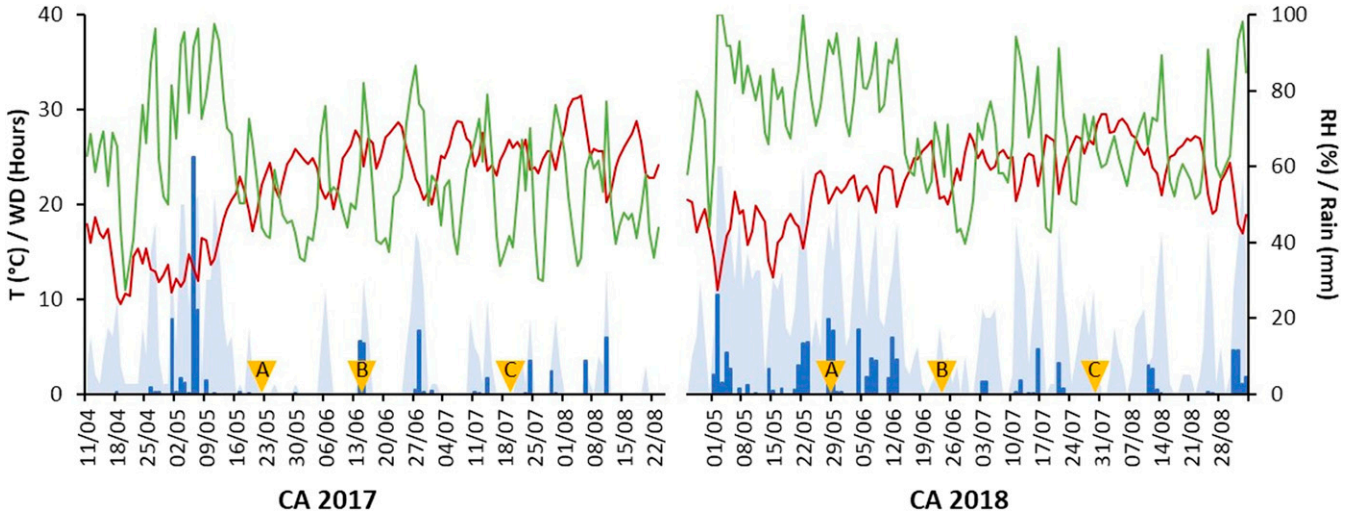

Fig. 2. Weather conditions in the vineyard of Castell'Arquato (CA) in 2017 and 2018 (experiment 2). Daily values of temperature (T; red line; in degrees Celsius), RH (green line; in percentage), rain (blue bars; in millimeters), and wetness duration (WD; light blue area; in hours) between the grape growth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

GS53 and full flowering (GS65) in both years (100 mm of rain and 200 h of wetness in 2015; 200 mm of rain and 108 h of wetness in 2016); in the following period, both rain and hours of wetness were lower (Fig. 1, I). At MA, the experimental period was 80 days in 2015 and 113 days in 2016. In both years, >200 mm of rain and >400 h of wetness were registered between inflorescence development (GS53) and prebunch closure (GS77); the later period was drier until GS83 (Fig. 1, II). 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 (>300 mm in both years), and 355 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. 1, III).

The average number of *B. cinerea* conidia per gram in the NT bunch trash was significantly higher in 2015 than in 2016 ($4.34 \pm 0.94 \times 10^5$ versus $9.67 \pm 1.51 \times 10^2$, respectively; $P < 0.001$), 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 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 3).

In each year, the number of *B. cinerea* conidia produced on bunch trash collected 1 week after flowering, prebunch closure, or veraison was affected by the interaction between vineyard and timing of FUN 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 FUN applications (models 2, 4, 6, 8, 10, and 12 in Table 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 3), which was reduced by FUN applied at A compared with NT ($P = 0.030$) with an estimated efficacy ranging from 0.53 to 0.68 (Fig. 3, I). The second case refers to the sporulation of bunch trash samples collected 1 week after veraison in vineyard CA in 2015 (Table 3), which was reduced by FUN application at A, B, C, or ABC ($P < 0.001$) with estimated efficacy ranging from 0.78 to 0.94 (Fig. 3, II). The third case refers to the sporulation of bunch trash samples collected 1 week after veraison in vineyard CO in 2016 (Table 3), which was reduced by FUN application 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. 3, III). 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 3).

When all of the sporulation data in Table 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 after an 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 ($\pm 1.994) \times 10^{-6}$ for the explanatory variable (i.e., conidia per gram of bunch trash; *b*); therefore, probability =0.5 when the sporulation potential is 10.423×10^5 conidia per gram. This means that, when the sporulation potential of *B. cinerea* on bunch trash was lower than this value, the probability that an FUN application was effective was <0.5.

Colonization of bunch trash by *B. cinerea* as affected by product and timing of application (experiment 2). Weather conditions differed between the 2 years of experiment 2 (Fig. 2). The experimental period (i.e., between inflorescences clearly visible and veraison; GS53 and GS83, respectively) was 111 days long in 2017 and 87 days

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

[a] One week after application at A (full flowering) (GS65 of Lorenz et al. 1995), B (prebunch 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* value) when comparing models with the same dataset.
[e] Timings of FUN applications were A (full flowering) (GS65 of Lorenz et al. 1995), B (prebunch 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.) at the label dose (800 g/ha).
[f] Vineyards were Castell'Arquato (CA), Cormons (CO), and Mandriole (MA).

Table 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 and model[a]	Factors[b]	Residual deviance[c]	df[d]	Null deviance	df[e]	AIC[f]
2017						
1	Product[g]	66.53	76	84.65	78	94.65
2	Product + Timing[h]	61.32	73	84.65	78	91.52
3	Product × Timing	55.09	67	84.65	78	99.40
2018						
4	Product	44.57	77	46.62	79	98.69
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, a fixed factor with timing of application, or an interaction of both factors.
[c] 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).
[d] Residual degrees of freedom.
[e] Residual degrees of freedom for the null model.
[f] AIC, Akaike's information criterion.
[g] Products were i) fungicide, a commercial mixture of fludioxonil (25%) and cyprodonil (37.5%; Switch; Syngenta Italia S.p.A.) at the label dose (800 g/ha); ii) botanicals, a commercial mixture of eugenol (3.2%), geraniol (6.4%), and thymol (6.4%; 3LOGY; Sipcam Italia S.p.A.) at the label dose (4,000 ml/ha); and iii) biological control agents (BCAs) *Aureobasium pullulans* (Botector; Manica S.p.A.) at full flowering and veraison and *Trichoderma atroviride* (Vintec; Belchim Crop Protection Italia S.p.A.) at prebunch closure (Pertot et al. 2017). Both BCA products were sprayed at the label dose (400 and 1,000 g/ha, respectively).
[h] Timings of applications were A (full flowering) (GS65 of Lorenz et al. 1995), B (prebunch closure; GS77), and C (veraison; GS83).

long in 2018 (Fig. 2). From GS53 to GS65, the average temperature was higher in 2018 than in 2017 (Fig. 2). In both years, the rain fell mostly between GS53 and GS77 (147.6 mm in 2017 and 204.4 mm in 2018), but the number of hours of wetness was higher in 2018 (608 h) than in 2017 (343 h) (Fig. 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 in 2017 (93 h of wetness) (Fig. 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 2). The selected model 2 showed no overdispersion (D/df = 0.84), and the assumptions of independence of errors, normality, and homoscedasticity of the residuals were confirmed (data 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 with NT ($P = 0.011$); BCA efficacy was not significantly different from that of FUN ($P = 0.573$), whereas BOT efficacy was significantly lower than that of FUN or BCA ($P < 0.001$) (Fig. 4, I). The application at A had the same efficacy as applications at ABC ($P = 0.468$), whereas efficacy was lower for applications at B and C than at ABC ($P = 0.075$ and $P = 0.057$, respectively) (Fig. 4, I). 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. 4, I).

In 2018, models 4 and 5 had the lowest AIC values (Table 2); no overdispersion 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 (data 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

Table 3. Number of *Botrytis cinerea* conidia produced per gram of bunch trash collected from plots that were not treated with fungicide in three vineyards in 2015 and 2016 (experiment 1)

	Sampling time: 1 week after		
Year and vineyard[a]	**Flowering (GS65)**	**Prebunch 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] Vineyards were Castell'Arquato (CA), Cormons (CO), and Mandriole (MA).
[b] Values and 95% confidence intervals of the numbers of conidia produced by *B. cinerea* on bunch trash after incubation at 20°C and 100% RH for 5 days estimated by transforming the parameters of the generalized linear models (GLMs) on their response scale (Table 1 shows the GLM fit).

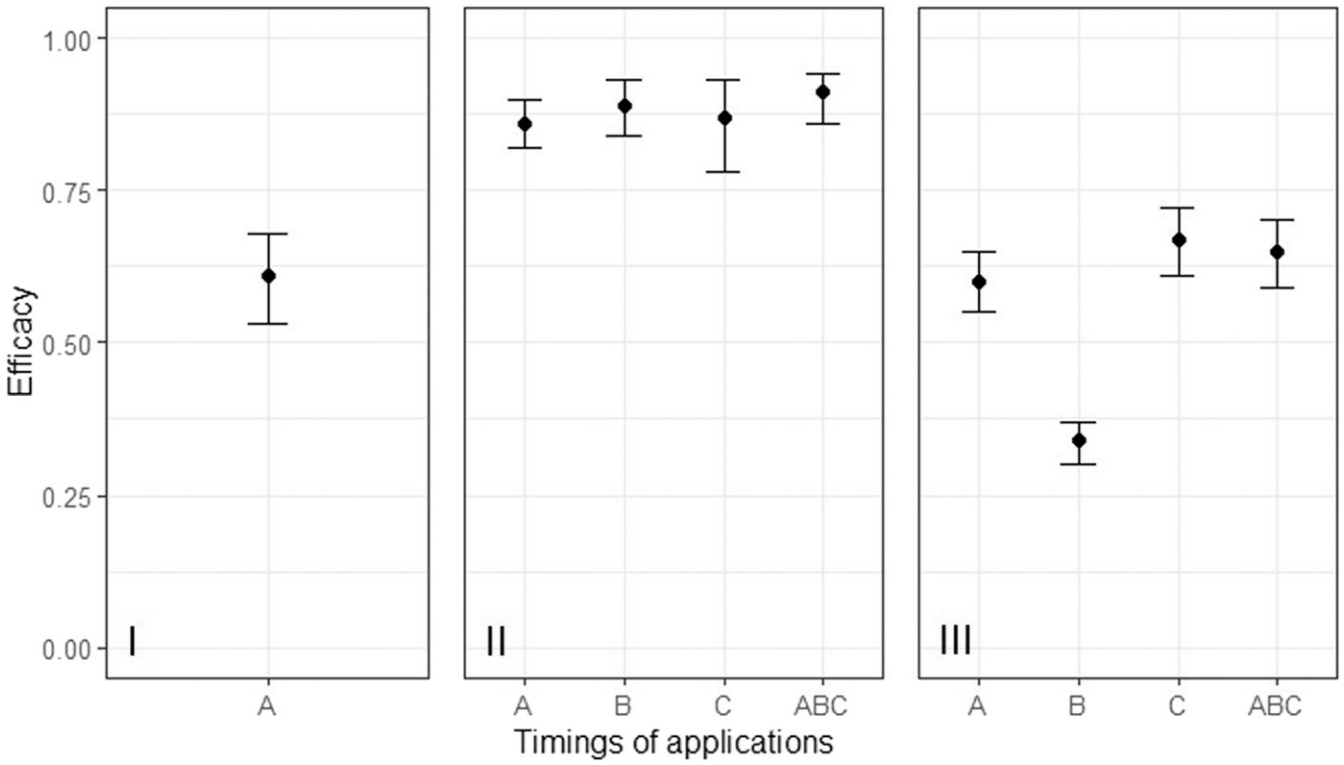

Fig. 3. Efficacy of a fungicide applied at different timings in the growing season in reducing the sporulation potential of *Botrytis cinerea* on bunch trash (experiment 1). Bunch trash samples were collected 1 week after flowering in vineyard Mandriole 2015 (I) and 1 week after veraison in vineyards Castell'Arquato in 2015 (II) and Cormons in 2016 (III). The fungicides were applied at A (full flowering) (GS65 of Lorenz et al. 1995), B (prebunch 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 95% confidence intervals.

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 with 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 they 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. 4, II).

Discussion

B. cinerea has a complex lifecycle 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 prebunch closure has been recommended (Corvi and Tullio 1980; Pérez-Marín 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 control agents or botanicals.

This research used field experiments and laboratory assessments to determine how early season applications of fungicides, biological control agents, and botanicals affect *B. cinerea* colonization of and

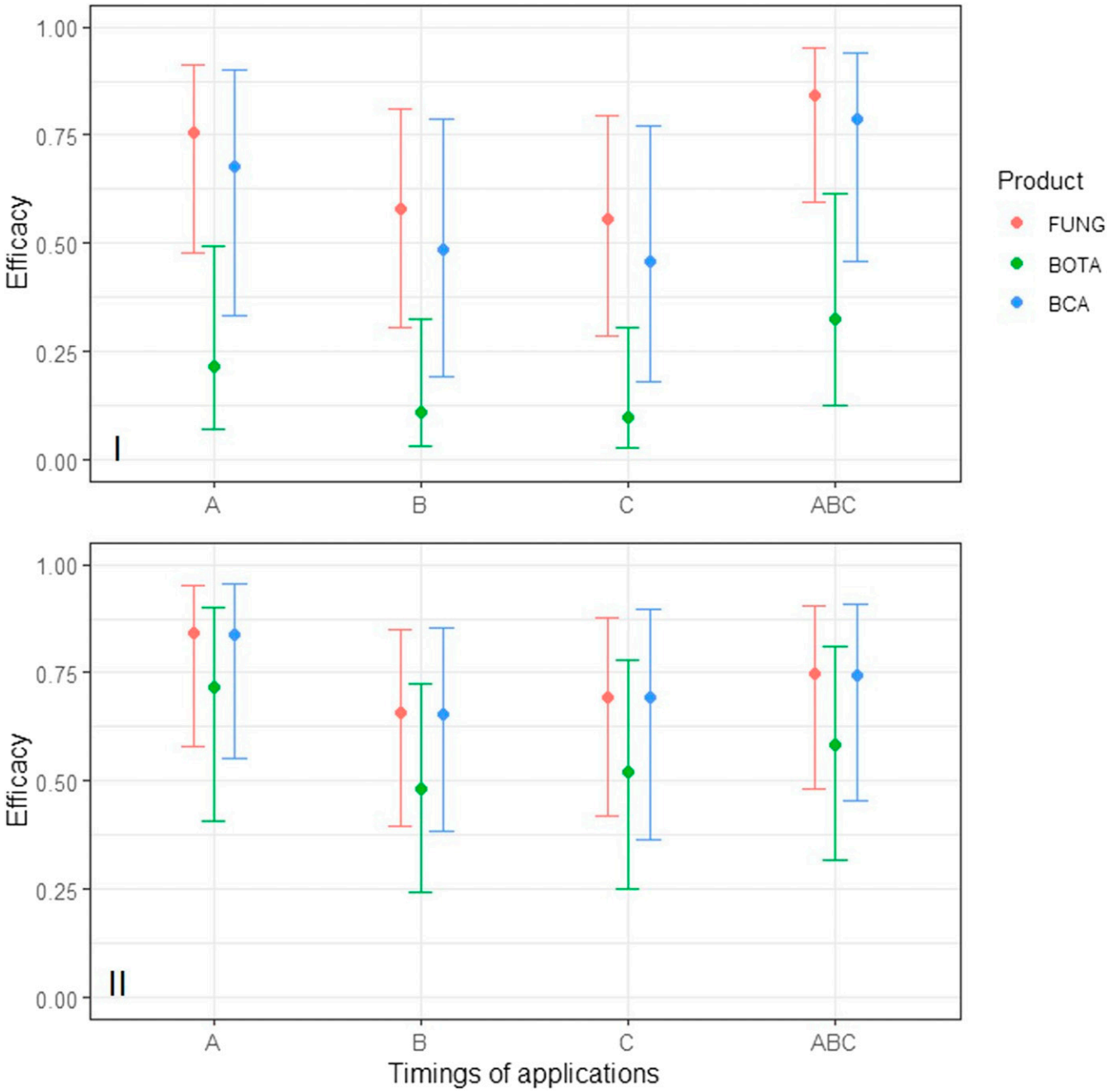

Fig. 4. Efficacy of different products applied at different timings in reducing bunch trash colonization by *Botrytis cinerea* (experiment 2). Bunch trash samples were collected 1 week after veraison in vineyard Castell'Arquato in 2017 (I) and 2018 (II). Products were applied at A (full flowering) (GS65 of Lorenz et al. 1995), B (prebunch closure; GS77), C (veraison; GS83), or ABC. The products were fungicide (FUNG; a commercial mixture of fludioxonil [25%] and cyprodonil [37.5%] at 800 g/ha), botanicals (BOTA; a commercial mixture of eugenol [3.2%], geraniol [6.4%], and thymol [6.4%] at 4,000 ml/ha); and biological control agents (BCAs; *Aureobasium pullulans* and *Trichoderma atroviride* at 400 and 1,000 g/ha, respectively). Bars indicate 95% confidence intervals.

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 this research. Instead, this 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 BCAs applied at three times: 1 to 5% flowering, 80% flowering, and prebunch closure. Using laboratory incubations with optimal conditions for colonization and sporulation, the authors found that the BCAs reduced the colonization of the bunch trash but not the sporulation potential on the bunch trash. Experiment 2 in this study also revealed that application of BCA, 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 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) 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 nontreated 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). 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 metaanalysis of 116 studies (González-Domínguez et al. 2019a, b). 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 this study indicate that the efficacy of application at A is also owing to a reduction in bunch trash colonization and 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 the fact that FUN applications significantly reduced *B. cinerea* sporulation in only 3 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, although 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 BCAs: *A. pullulans*, which was applied at A and C, is a good competitor for nutrients and can prevent germination of *B. cinerea* conidia; *T. 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); this 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 this study, Rotolo et al. (2018) did not obtain satisfactory BBR control on table grapes at harvest. Nevertheless, BOTs are thought to have potential for controlling *B. cinerea* (Nguyen et al. 2013; Ribera et al. 2008), and additional studies are needed to determine whether applications of BOT at A can reduce latent infections (i.e., pathways 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 versus 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. Therefore, 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 been developed (González-Domínguez et al. 2015). This model, which is currently integrated in a decision support system (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: between i) 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). These tools (the model and the qPCR assay) combined with the findings of this study could improve BBR management in vineyards.

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