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# The Colonization of Grape Bunch Trash by Microorganisms for the Biocontrol of *Botrytis cinerea* as Influenced by Temperature and Humidity

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**Abstract:** Six commercial biocontrol agents (BCAs: *Aureobasidium pullulans*, *Bacillus amyloliquefaciens*, *B. amyloliquefaciens* plantarum, *B. subtilis*, *Metschnikowia fructicola*, or *Trichoderma atroviride*) were applied to bunch trash that was then incubated at one of five temperatures (T, 15, 20, 25, 30, and 35 °C) and one of five relative humidity levels (RH, 60, 80, 90, 95, and 100%). After 1 to 13 days of incubation (BCA colonization period), the number of colony forming units (CFUs) was assessed. The colonization of bunch trash in response to T/RH conditions and BCA colonization period differed among the BCAs; the coefficients of variation among the BCAs ranged from 104.6 to 397.7%. Equations were developed that accounted for the combined effects of the T, RH, and BCA colonization period on BCA colonization of bunch trash. Assuming that the equations, which had an $R^2 > 0.87$, correctly predict BCA growth under field conditions, they would help farmers select the BCA to be used for a specific application based on weather conditions at the time of treatment and in the following days. The equations would also help predict how long an early season BCA application remains effective and thereby help farmers decide whether and when a second BCA application may be needed.

**Keywords:** grey mold; biological control; biological control agents; integrated pest management; modeling; *Vitis vinifera*

## 1. Introduction

Botrytis bunch rot (BBR) is an economically important disease of grapevine (*Vitis vinifera* L.) and is caused by the fungus *Botrytis cinerea* Pers.:Fr (anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel) [1]. *Botrytis cinerea* can develop as a saprophyte, necrotroph, or parasite on multiple vine organs including leaves, green shoots and rachides, flowers and flowering residues, and berries. *B. cinerea* also has multiple infection pathways, with infections mainly occurring from flowering to fruit set and after veraison [1–5].

BBR control is traditionally based on the application of fungicides at four grape growth stages (GS): A, end of flowering (GS69; [6]); B, pre-bunch closure (GS77); C, veraison (GS83); and D, before harvest (before GS89) [7,8]. These routine applications of fungicides make it possible to control all *B. cinerea* infection pathways. At flowering, conidia germinate and infect the flower styles and ovules (infection pathway I of Elmer and Michailides [1]), stamens and petals (pathway IIa), or the fruit pedicel (pathway IIb). Starting from flowering, the pathogen also saprophytically colonizes the bunch trash (calyptras, dead stamens, aborted flowers and berries, and tendrils) retained within the developing bunches (pathway III). Under favorable conditions, the bunch trash colonized by *B. cinerea* repeatedly produces conidia that can infect the ripening berries (pathway IV). An increased interest in the reduction in

the use of chemical fungicides for BBR control in vineyards has stimulated the search for natural products that can substitute for or complement the chemical fungicides. These natural products include microbial biocontrol agents (BCAs) like yeasts, fungi, and bacteria [9–11].

BCAs have been studied for their efficacy in reducing both the colonization of bunch trash by *B. cinerea* [12,13] and BBR on ripening berries [14–16]. In these studies, BCAs have been found to be effective to different degrees in controlling *B. cinerea* by different mechanisms of action, including: (i) competition for space and nutrients, which limits pathogen growth and sporulation; (ii) antibiosis, i.e., the production of metabolites that limit the germination and growth of the fungus; and (iii) mycoparasitism, i.e., the invasion of the pathogen's hyphae by the BCA [16–22].

Inconsistent efficacy across seasons and local agronomic conditions [14,23], however, has hindered the widespread use of BCAs for BBR control. One possible reason for the inconsistency is that BCA efficacy is closely related to pathogen and BCA growth dynamics [21,24,25], which are influenced by environmental conditions. Temperature and humidity, for instance, affect the mycelial growth, conidial germination, and sporulation of *B. cinerea* [3,4], as well as the survival, establishment, and growth of the BCA [26–29]. Other factors affecting the establishment and efficacy of BCAs are the biochemical characteristics (pH, presence of nutrients, etc.) of the plant surfaces (e.g., inflorescences, bunch trash, and berries at different maturation stages), which vary during the season, and the period of time after BCA application, which can influence the degree to which the BCA has depleted its nutritional resources (caused by natural decay and/or colonization) [26]. It follows that a successful integration of BCAs into a BBR management strategy [30] with the aim of reducing *B. cinerea* colonization of and sporulation on bunch trash requires an understanding of the pathogen's and BCA's environmental requirements on the bunch trash at the time of application and in the following days.

In the current research, we investigated the effects of temperature and relative humidity on the colonization of bunch trash by six commercial biocontrol products (each with a different BCA). Although conducted in growth chambers, the research included five temperature and five relative humidity levels that represented a wide range of conditions relevant to vineyards.

## 2. Materials and Methods

### *2.1. Plant Material*

Bunch trash was collected in 2018 and 2019 in an 11-year-old vineyard (in 2018) located at Castell'Arquato (44°51'26.1''N 9°51'20.7''E, 400 m at sea level), in North Italy. The vineyard was representative of the area; planted with cv. Merlot, and the vines were trained using a Guyot system; the within- and between-row spacing were 1.0 and 2.3 m, respectively. Powdery and downy mildews were controlled according to an integrated pest management (IPM) program [31], and the fungicides applied were not effective against *B. cinerea*. The area is conducive to BBR and growers usually spray specific fungicides twice per season, at pre-bunch closure and during berry ripening.

Bunch trash was collected at the end of flowering (GS69 of Lorenz et al. [6]) by gently shaking 100 random bunches, and was immediately transported in a cooler bag to the laboratory. After it was dried in an oven at 80 °C for 72 h, the bunch trash was divided into 0.1-g aliquots, which were autoclaved at 120 °C for 20 min to kill microorganisms. Finally, each aliquot of bunch trash was uniformly arranged on a sterile paper dish on the bottom of a Petri plate (5.5 cm in diameter, one aliquot/paper dish per Petri plate).

### *2.2. Treatment of Bunch Trash with BCAs*

Six commercial BCAs were used (Table 1). These products were dispersed in double-distilled sterile water (pH 6.5) at the label dose. A micropipette was used to uniformly distribute the BCA suspensions on the bunch trash in the Petri plates (1 mL of suspension per Petri plate). The viability of the BCAs was confirmed by plating the product suspensions on PDA (potato dextrose agar, Biolife Italiana S.r.l., Milano, Italy).

**Table 1.** Biocontrol agents (BCAs) used in the experiment.

| Active Ingredient | Commercial Product (Acronym) | Producer | Label Dose (g/ha) |
|---|---|---|---|
| *Bacillus amyloliquefaciens* D747 | Amylo-X (AMY) | CBC S.r.l. | 2000 |
| *Aureobasidium pullulans* DMS 14941-14940 | Botector (BOT) | Manica S.p.A. | 400 |
| *Metschnikowia fructicola* | Noli (NOL) | Koppert Italia | 2000 |
| *Bacillus subtilis* QST 713 | Serenade max (SER) | Bayer S.p.A. | 3000 |
| *Bacillus amyloliquefaciens* FZB24 | Taegro (TAE) | Syngenta | 370 |
| *Trichoderma atroviride* SC1 | Vintec (VIN) | Belchim S.p.A. | 1000 |

Petri plates were then placed on a metallic grid inside metal boxes (20 × 15 cm; 3 plates per box) and incubated at different regimes of temperature (T=15, 20, 25, 30, or 35 °C) and relative humidity (RH=60, 80, 90, 95, or 100%), with a 12 h photoperiod. Different RH values were obtained by placing 200 mL of double-distilled water alone or double-distilled water containing appropriate concentrations of glycerol [32] on the bottom of the metal boxes. The boxes were sealed in plastic bags to maintain the desired atmospheric conditions. The true T and RH inside the boxes were determined with data loggers (Tinytag Plus 2, Gemini Data Loggers, Chichester, UK). There were 3 Petri plates (replicates) for each T/RH) regime. The experiment was repeated once (Figure 1).

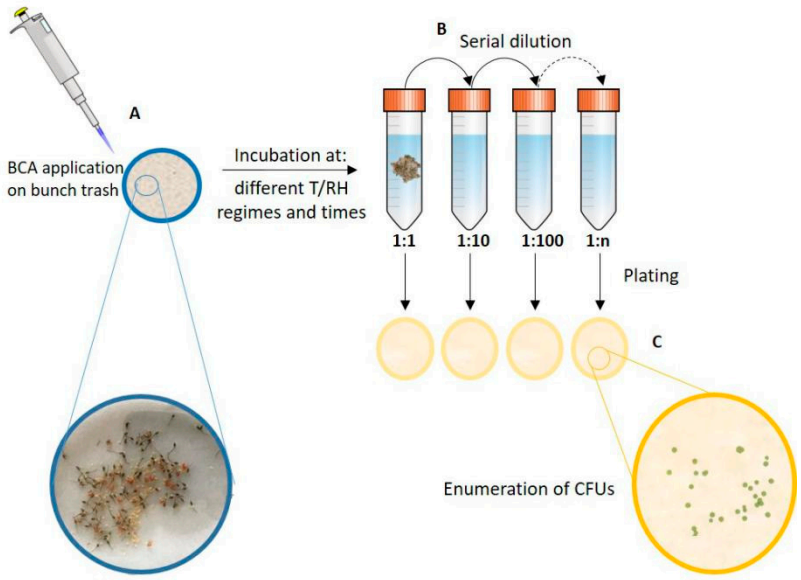


**Figure 1.** Bunch-trash aliquots were arranged in Petri plates (one aliquot per plate) over a wet filter paper and inoculated with the BCA suspension using a micropipette (**A**). Petri plates were then incubated at different temperature/relative humidity regimes. After 1, 3, 6, 9, and 13 days, the bunch trash was placed in a 15-mL flask containing 10 mL of sterile double-distilled water, shaken by hand and serially diluted from 1:1 to 1:n, where 1:n is the dilution making the colony enumeration possible (**B**). The number of colony forming units (CFUs) was finally determined by plating 100 µL of each dilution on potato dextrose agar (PDA) plates (**C**), and expressed as CFUs per g of bunch trash.

### *2.3. Assessment of Colony Forming Units (CFUs)*

At 1, 3, 6, 9, and 13 days after treatment with BCAs, the bunch trash from each Petri plate was placed in a 15-mL flask containing 10 mL of sterile double-distilled water. The flask was briefly shaken by hand to wash microorganisms off the surface of the trash. After that, the number of colony forming units (CFUs) per g of bunch trash was determined by serially diluting the suspension of

microorganisms (without pieces of trash). A quantity of 100 µL of each serial dilution was plated on PDA plates (9 cm in diameter) and incubated at 25 °C for 48 h (Figure 1).

### *2.4. Data Analysis*

The CFU data were subjected to a factorial analysis of variance (ANOVA), in which the factors were BCA treatment (six BCAs), T/RH regime (combinations of T and RH), and the number of days that bunch trash were kept in the incubator (BCA colonization periods: 1, 3, 6, 9, or 13 days). The experimental design was a split-split, with BCAs as the main plots, T/RH regimes as the split plots, and BCA colonization periods as the split-split plots. The CFU data were ln(x + 1) transformed before the ANOVA to make variances homogeneous. The transformed CFU data followed a normal distribution according to the Shapiro–Wilk test ($p > 0.05$).

To assess the variability in the number of CFUs as affected by T/RH regime and colonization period within each BCA, coefficients of variation (CV, in %) [30], or relative standard deviations (SDs) were calculated as the ratio of the SD to the mean; the higher the CV, the greater the variability generated by T/RH regimes and the length of the BCA colonization period.

To investigate the response of each BCA to the different T/RH regimes, the numbers of CFUs were first rescaled from 0 to 1 as follows: the number of CFUs were first divided by the number of days of incubation, to obtain the average number of CFUs per day of incubation; each value was then divided by the highest value found in the experiment for each of the BCAs. For BOT, for example, the highest number of CFUs was $3.35 \times 10^8$, which occurred at 25 °C, 100% RH, and with 3 days of incubation; the average number of CFUs per day was $1.12 \times 10^8$ (i.e., $3.35 \times 10^8/3$). At 15 °C and 100% RH, in contrast, the number of CFUs was the highest ($2.75 \times 10^7$) with 13 days of incubation, and the average number of CFUs per day was $2.81 \times 10^6$ (i.e., $2.75 \times 10^7/13$). Rescaled values for the two cases were 1.000 ($1.12 \times 10^8/1.12 \times 10^8$) and 0.025 ($2.81 \times 10^6/1.12 \times 10^8$), respectively.

Rescaled CFU values were then fit to equations that accounted for the effect of T/RH regimes. Two kinds of equations (either (1) or (3)) were used depending on the response of CFU to RH; these equations were selected from a set of candidate equations using the Akaike information criterion (AIC), which is an estimator of the relative quality of statistical models for a given set of data [33] (not shown).

Equation (1) was used for BCAs for which the optimal RH was 100%:

$$Y = \left[a\ \mathrm{Teq}^b\ (1\ -\ \mathrm{Teq})\right]^c \left[1 - d\left(1 - e^{\mathrm{VPD}}\right)\right] \quad (1)$$

where Y is the rescaled number of CFUs; Teq is the temperature equivalent, defined as Teq = (T − Tmin)/(Tmax − Tmin), where T is the temperature regime (in °C) and Tmin and Tmax are cardinal temperatures (in °C); and VPD (in kPa) is the vapor pressure deficit calculated as follows [34]:

$$\mathrm{VPD} = 0.61121\ \exp\left((18.678\ -\mathrm{T}/234.5)\ (\mathrm{T}/(257.14 + \mathrm{T}))\right)\ (1 - \mathrm{RH}/100) \quad (2)$$

where RH is relative humidity (in %).

Equation (3) was used for BCAs for which the optimal RH was <100%:

$$Y = \left[a\ \mathrm{Teq}^b\ (1\ -\ \mathrm{Teq})\right]^c \left\{E'\left\{\exp\left[\left(\mathrm{VPD}_{eq} - f\right)g/(h+1)\right\}/\{1 + \exp[(\mathrm{VPD}\ -\ f)g]\}\right\}\right. \quad (3)$$

where Y is the rescaled number of CFUs; Teq is as previously described; VPDeq is the vapor pressure deficit equivalent, defined as $\mathrm{VPD}_{eq}$ = −(VPD − VPDmin) * 10, where VPD is as previously described and VPDmin is equal to 1.584; and $E'$ is calculated as follows:

$$E' = E[(h+1)/h]h^{1/(h+1)} \quad (4)$$

The first term of Equations (1) and (3), $[a\mathrm{Teq}^b(1 - \mathrm{Teq})]^c$, accounts for the effect of temperature according to the bell-shaped curve of Analytis [35], with parameters $a$, $b$, and $c$ defining the top,

symmetry, and size of the curve, respectively. The second term of Equation (1), $[1 - d(1 - e^{VPD})]$, accounts for the combined effect of T and RH (as VPD) on Y (the rescaled number of CFUs) according to an asymptotic equation, where 1 is the maximum attainable value for Y, $d$ is the value for Y at VPD = 0, and $e$ is proportional to the relative rate of decrease for Y when VPD increases. The second term of Equation (3), $\{E\ \{\exp[(VPD_{eq} - f)g/(h + 1)\}/\{1 + \exp[(VPD - f)g]\}\}$, accounts for the effect of T and RH (as VPD) on Y according to a Weibull equation [36]. The equation defines a unimodal curve in which the response declines from 1, which is the maximum attainable value for Y, and approaches a lower limit of 0 as VPD increases or decreases from the optimum. The intrinsic rate of decline and degree of asymmetry in VPD response are described by the parameters $g$ and $h$, respectively.

To investigate the time (in days) required by each BCA from inoculation to maximal colonization, the highest number of CFU at any temperature and RH was divided by the corresponding number of days and then rescaled from 0 to 1 as described before; only the fit for 100% RH was shown in this paper for simplicity. The rescaled values were then fit to the following equation derived from Reed et al. [37] and Wadia and Butler [38]:

$$Y = m \Big/ \left[ \left( \frac{T - Tmin}{Topt - Tmin} \right) \times \left( \frac{Tmax - T}{Tmax - Topt} \right)^{\left( \frac{Tmax - Topt}{Topt - Tmin} \right)} \right] \quad (5)$$

where $m$ is the minimum length of the colonization period (number of days) required for the maximal BCA colonization at the optimal temperature (Topt, °C); T, Tmin, and Tmax are as previously described.

To investigate BCA colonization as a function of time (days) at the optimal temperature and 100% RH, the rescaled values were fit to the following equation derived from Peleg and Corradini [39]:

$$Y = N_0\ exp\left[ \left( \frac{t}{t_{cg}} \right)^{m_1} \right] \exp\left[ -\left( \frac{t}{t_{cd}} \right)^{m_2} \right] \quad (6)$$

where $N_0$ is the initial population (i.e., the population at time 0), $t_{cg}$ and $t_{cd}$ represent the characteristic times of the growth and the decay, respectively, had they been unimpeded; $m_1$ and $m_2$ are coefficients that account for the steepness of the curve, respectively.

The following equation parameters were estimated using the function *nls* of the "stats" package of R software [40]: $a$ to $e$ in Equation (1), $a$ to $c$ and $f$ to $i$ in Equation (3), $m$, Tmin, Topt, and Tmax in Equation (5), and $t_{cg}$, $t_{cd}$, $m_1$, and $m_2$ in Equation (6). The parameterized equations were evaluated for goodness-of-fit based on the adjusted $R^2$, the root mean square error (RMSE), the coefficient of residual mass (CRM), and the concordance correlation coefficient (CCC) [41,42]. The adjusted $R^2$ was estimated by conducting a linear regression between the observed values and the model predicted values; the linear regression was conducted with the *lm* function of the R "stats" package [40]. The RMSE was obtained using the *rmse* function of the R "modelr" package [43]. The CCC was obtained using the CCC function of the R "DescTools" package [44]. In brief, RMSE represents the average distance of real data from the fitted line, and CRM is a measure of the tendency of the equation to overestimate or underestimate the observed values (a negative CRM indicates a tendency of the model toward overestimation) [42]. CCC is the product of two terms: the Pearson correlation coefficient and the coefficient Cb, which indicates the difference between the best fitting line and the perfect agreement line (CCC = 1 means perfect agreement) [45].

## 3. Results

The ANOVA showed a significant effect of the main factors BCA, T/RH regime, and BCA colonization period (all at $p < 0.001$), which explained 41.2, 26.5, and 5.9%, respectively, of the total variance in number of CFUs. The interactions BCA × T/RH regime, BCA × colonization period, T/RH regime × colonization period, and BCA × T/RH regime × colonization period were also significant (all at $p < 0.001$), and accounted for 25.6, 15.6, 18.6, and 33.2% of the total variance in number of CFUs,

respectively. These data indicate that the number of days of BCA colonization of bunch trash and the T/RH regime during this time significantly affected the BCA colonization level, but that the response to T/RH regime and colonization period differed among the BCAs.

The coefficient of variation (Figure 2) of CFU data ranged from 104.6% (for NOL) to 359.7% (for BOT), which indicated that all of the BCAs were sensitive to T/RH regimes and the length of the colonization period, with some of them being more sensitive than others.

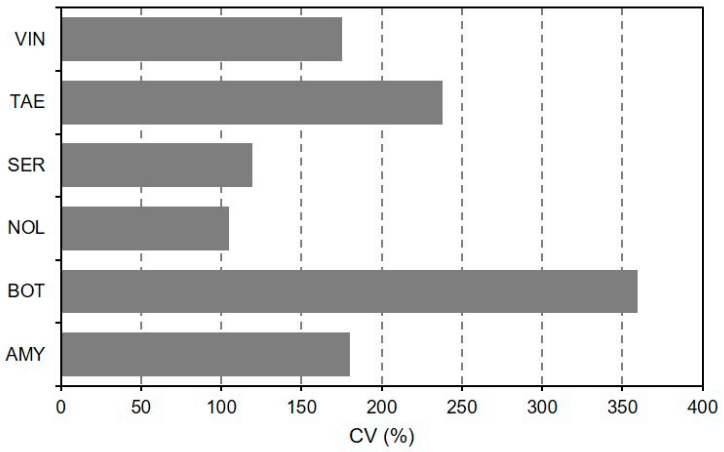


**Figure 2.** Coefficient of variation (CV, %) for the numbers of colony forming units (CFUs) of the six BCAs that developed on bunch trash. After it was treated with one of six BCAs, the bunch trash was subjected to different temperature and relative humidity regimes for different BCA colonization periods. Full BCA names and background information are provided in Table 1.

Relative colonization by BCAs as affected by length of BCA colonization period and T/RH regime is shown in Figure 3. The BCAs differed in the time required to achieve maximal colonization, i.e., some BCAs were faster than others (Figure 3A). The responses of BCA colonization to specific T and RH (both considered as main effects) differed among the BCAs (Figure 3B,C). The responses of BCA colonization to T were characterized by a bell-shaped pattern for all of the BCAs (Figure 3B); the responses of BCAs colonization to RH differed among BCAs, with some producing bell-shaped patterns and others producing exponentially increasing patterns (Figure 3C).

Equation (1) provided a good fit for the relative colonization data for BOT, SER, and TAE, with $R^2 > 0.89$, RMSE $\leq 0.105$, CRM $\leq 0.069$, and CCC $> 0.945$ (Table 2). Similarly, Equation (3) provided a good fit for the relative colonization data for AMY, NOL, and VIN, with $R^2 > 0.92$, RMSE $\leq 0.09$, CRM $\leq 0.127$, and CCC $> 0.902$ (Table 2). This indicated that solving Equation (1) or Equation (3) for any combination of T (between 15 and 35 °C) and RH (between 60 and 100%) provided a reliable prediction of the relative colonization of bunch trash by the BCAs. The contour plots of the relative colonization of bunch trash by the six BCAs are shown in Figure 4; the relative colonization values were obtained by solving Equation (1) or Equation (3) with the parameters of Table 2. The relative colonization values used for Figure 4 were grouped into five categories to clarify the different responses of the BCAs to T/RH, from low to high colonization, as follows: low, 0 to 0.2 relative colonization; medium-low, 0.2 to 0.4; medium, 0.4 to 0.6; medium-high, 0.6 to 0.8; and high: 0.8 to 1. For TAE, high relative colonization required a high level of relative humidity (Figure 4E). BOT (Figure 4B) and NOL (Figure 4C) were able to colonize the bunch trash at lower levels of RH than the other three BCAs. Relative colonization was highest at 90 < RH < 95% for AMY (Figure 4A) and VIN (Figure 4F), and at 80 < RH < 95% for NOL (Figure 4C). Both SER (Figure 4D) and VIN (Figure 4F) were able to colonize bunch trash at a wider range of temperature than the other BCAs.

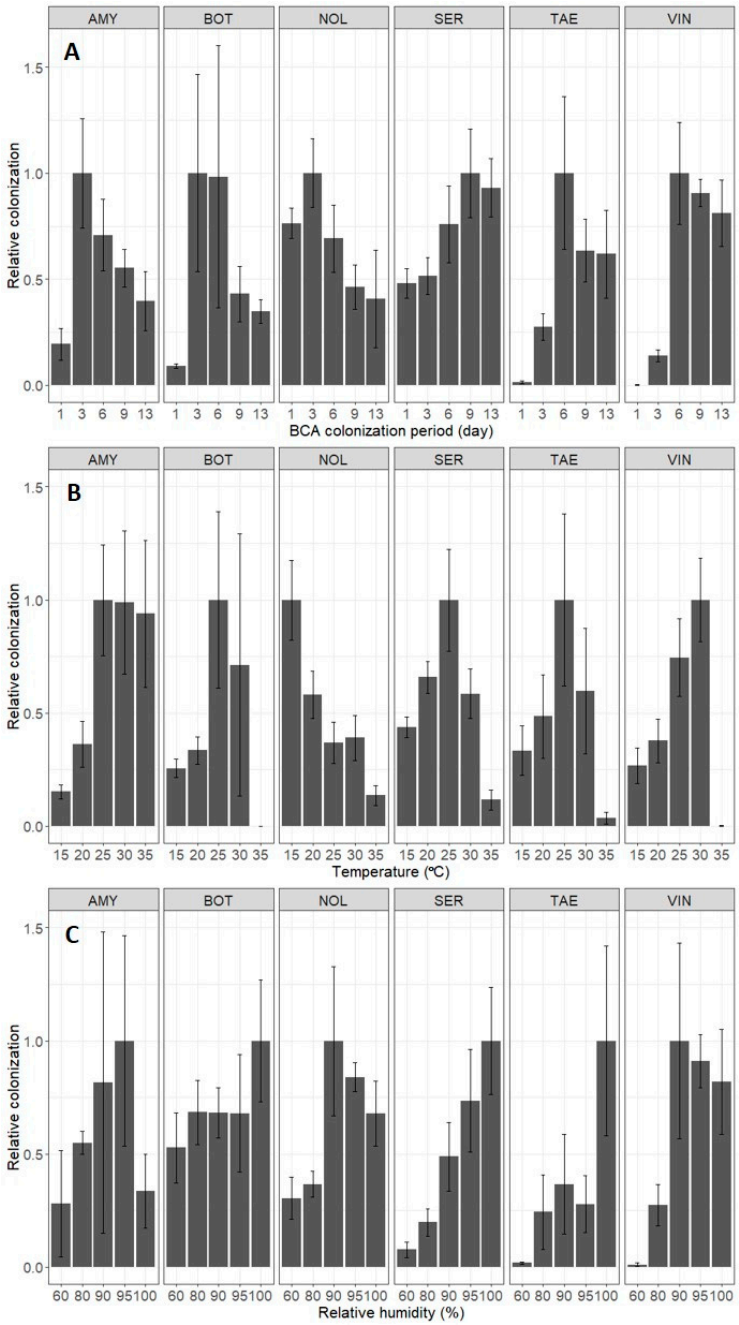


**Figure 3.** Relative colonization on bunch trash by the six BCAs as affected by (**A**) the length of the colonization period (in days), and by (**B**) the temperature and (**C**) relative humidity level that the bunch trash was subjected to after the BCA treatment. Bars are overall means of different T/RH regimes in (**A**) and of different numbers of days after the BCA treatment in (**B**) and (**C**); whiskers show standard error.

**Table 2.** Parameters of Equations (1) and (3) for the six BCAs, and statistics for goodness-of-fit to real data.

| BCA | Tmin/ Tmax [1] | Equation Parameters [2] | | | | | | | | Statistics [3] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *a* | *b* | *c* | *d* | *e* | *f* | *g* | *h* | $R^2$ | RMSE | CRM | CCC |
| AMY | 5/40 | 2.102 (0.118) | 3.000 (0.348) | 3.000 (0.207) | - | - | 15.40 (0.759) | 1.538 (1.174) | 8.00 (10.513) | 0.918 | 0.090 | −0.037 | 0.961 |
| BOT | 5/35 | 2.313 (0.076) | 2.511 (0.176) | 5.000 (1.279) | 0.686 (0.066) | 0.063 (0.043) | - | - | - | 0.892 | 0.105 | 0.069 | 0.945 |
| NOL | 0/37 | 3.641 (0.475) | 1.148 (0.217) | 5.643 (5.135) | - | - | 12.00 (4.356) | 1.00 (0.564) | 0.389 (1.206) | 0.956 | 0.071 | 0.127 | 0.902 |
| SER | 0/35 | 2.207 (0.070) | 2.712 (0.160) | 1.985 (0.361) | 0.991 (0.042) | 0.096 (0.022) | - | - | - | 0.990 | 0.032 | 0.007 | 0.995 |
| TAE | 0/35 | 2.091 (0.069) | 3.126 (0.243) | 6.350 (1.997) | 0.837 (0.084) | 0.0001 (0.0004) | - | - | - | 0.933 | 0.085 | 0.060 | 0.967 |
| VIN | 0/35 | 1.750 (0.238) | 54.762 (1.815) | 0.920 (0.704) | - | - | 12.00 (1.293) | 1.00 (0.234) | 0.269 (0.283) | 0.984 | 0.048 | 0.043 | 0.991 |

[1] Estimates of Tmin and Tmax used for the calculation of equivalents of temperature, Teq, used in Equations (1) and (3); [2] estimates of parameters of Equation (1): $Y = [a\ Teq^b\ (1 - Teq)]^c\ [1 - d(1 - e^{VPD})]$ and Equation (3): $Y = [a\ Teq^b\ (1 - Teq)]^c\ \{E'\{\exp[(VPD_{eq} - f)g/(h + 1)\}/\{1 + \exp[(VPD - f)g]\}\}$; [3] adjusted $R^2$, root mean square error (RMSE), coefficient of residual mass (CRM), concordance correlation coefficient (CCC).

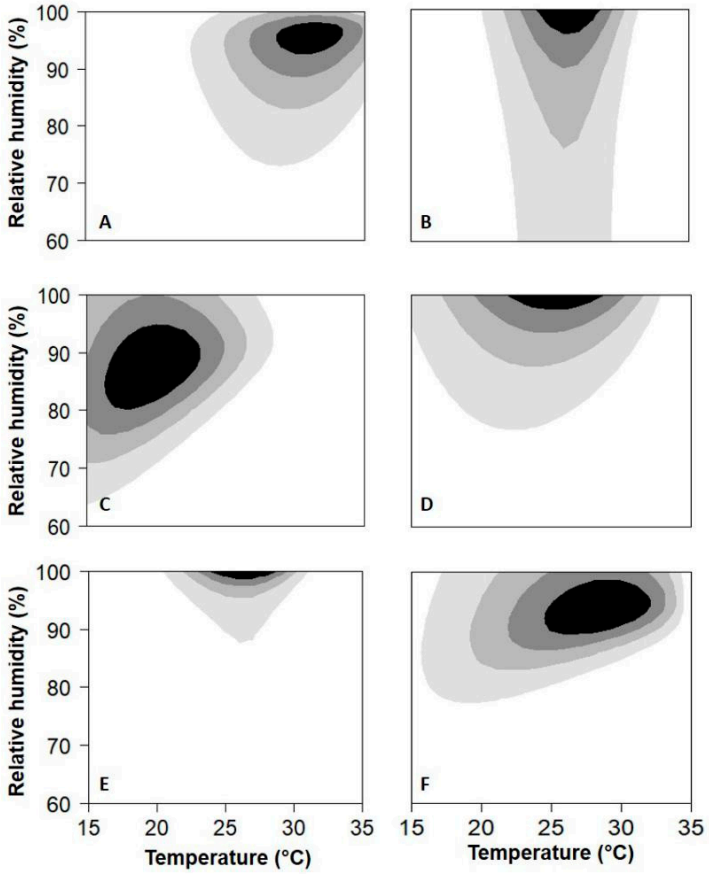


**Figure 4.** Relative colonization of bunch trash that was treated with one of six BCAs (**A**: AMY; **B**: BOT; **C**: NOL; **D**: SER; **E**: TAE; **F**: VIN, see Table 1) and that was then incubated at different temperatures (T) and with different relative humidity (RH) levels. The contour plots, which were generated with Equations (1) and (3), and the parameters listed in Table 2, identify five areas of relative colonization of bunch trash by the six BCAs: L (low, 0 to 0.2 relative colonization; the white area); ML (medium-low, 0.2 to 0.4; the light grey area); M (medium, 0.4 to 0.6; the medium grey area); MH (medium-high, 0.6 to 0.8; the dark grey area); and H (high: 0.8 to 1; the black area).

Equation (5) provided a good fit for the time required for the maximal colonization for all of the BCAs, with $R^2 > 0.87$, RMSE < 0.84, CRM < 0.07, and CCC > 0.86 (Table 3). This indicates that solving

Equation (5) for any temperature (between 15 and 35 °C) and RH = 100% provides a reliable prediction of the time required for the maximal colonization of each BCA on bunch trash. In Figure 5, Equation (5) and the parameters listed in Table 3 were used to calculate the number of days required by each BCA to reach the maximal colonization at the different temperatures, with RH = 100%. Overall, the time required for the maximal colonization was shorter at optimal temperatures, meaning that the length of the colonization period was temperature-dependent. Some BCAs, like AMY and TAE (Figure 5A,E), required fewer than 5 days at 35 °C and more than 13 days at temperatures <20 °C. NOL (Figure 5C) required fewer than 5 days to reach the maximal colonization across the whole temperature range (15–35 °C), indicating that the length of the colonization period was less temperature-dependent for this BCA.

**Table 3.** Parameters of Equation (5) for the six BCAs, and statistics for goodness-of-fit to real data.

| BCA | Equation Parameters [2] | | | | Statistics [3] | | | |
|---|---|---|---|---|---|---|---|---|
| | *m* [1] | Tmin | Topt | Tmax | $R^2$ | RMSE | CRM | CCC |
| AMY | 3 | 17.24 (2.95) | 35.00 (24.41) | 40.00 (89.08) | 0.976 | 0.514 | 0.018 | 0.992 |
| BOT | 3.8 | 11.80 (1.52) | 27.00 (2.40) | 30.14 (0.56) | 0.973 | 0.468 | −0.018 | 0.989 |
| NOL | 2.7 | 11.21 (1.83) | 27.25 (2.21) | 60.00 (61.90) | 0.959 | 0.212 | 0.005 | 0.984 |
| SER | 7 | 10.00 (7.99) | 24.75 (6.20) | 33.29 (18.68) | 0.874 | 0.695 | 0.069 | 0.862 |
| TAE | 4.5 | 16.09 (9.28) | 35.485 (41.61) | 50.00 (389.91) | 0.907 | 0.844 | −0.033 | 0.963 |
| VIN | 9 | 13.83 (2.41) | 27.57 (1.36) | 40.65 (6.98) | 0.999 | 0.012 | −0.014 | 0.993 |

[1] Estimates of the minimum length of colonization period (in days) required for the maximal colonization of BCA at the optimum temperature (Topt, °C); [2] estimates of parameters of Equation (5); [3] adjusted $R^2$, root mean square error (RMSE), coefficient of residual mass (CRM), concordance correlation coefficient (CCC).

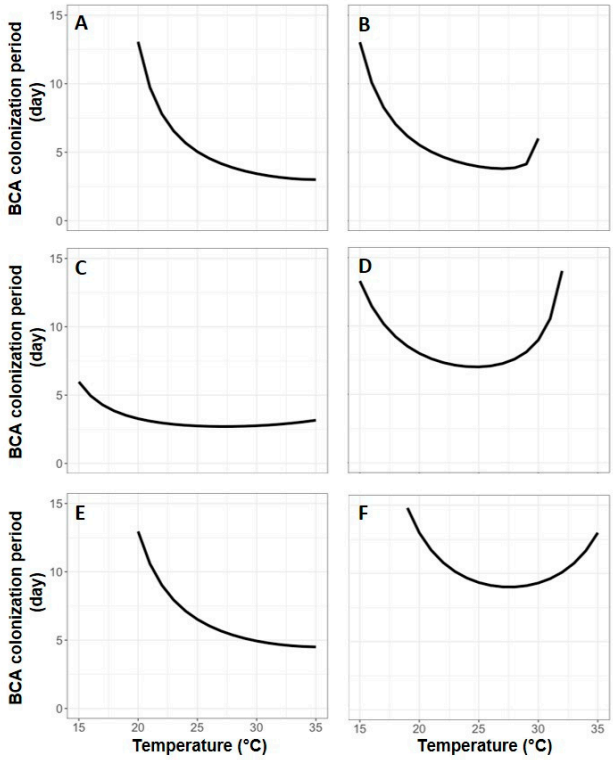


**Figure 5.** Effect of temperature on the length of the BCA colonization period required by the six BCAs (**A**: AMY; **B**: BOT; **C**: NOL; **D**: SER; **E**: TAE; **F**: VIN, see Table 1) to attain their maximal colonization of bunch trash. Bunch trash was treated with the BCAs listed in Table 1 and was then incubated in plates at different temperatures and with different RH levels (values in this figure are for RH = 100%); after 1, 3, 6, 9, and 13 days, the number of CFUs was assessed. Lines were drawn using Equation (5) and the parameters listed in Table 3.

Equation (6) provided a good fit for the characteristic times for growth and decay for all of the BCAs, with $R^2 > 0.79$, RMSE < 0.154, CRM < 0.277, and CCC > 0.87 (Table 4). This indicated that solving Equation (6) for optimal temperature and RH = 100% provides a reliable prediction of the time required for the growth and the decay of each BCA on bunch trash. In Figure 6, Equation (6) and parameters listed in Table 4 were used to calculate the relative colonization dynamics of bunch trash over time. As was the case for RH and temperature data, these data also indicate that the duration of colonization differed among the BCAs (Figure 6).

**Table 4.** Parameters of Equation (6) for the six BCAs, and statistics for goodness-of-fit to real data.

| BCA | Equation Parameters [1] | | | | Statistics [2] | | | |
|---|---|---|---|---|---|---|---|---|
| | $t_{cg}$ | $t_{cd}$ | $m_1$ | $m_2$ | $R^2$ | RMSE | CRM | CCC |
| AMY | 0.33 | 3.00 (14.758) | 0.621 (0.832) | 1.436 (2.391) | 0.919 | 0.091 | 0.031 | 0.949 |
| BOT | 0.50 | 3.573 (630.049) | 1.196 (31.143) | 5.00 (1482.421) | 0.942 | 0.078 | 0.207 | 0.966 |
| NOL | 0.30 | 3.602 (24.803) | 0.50 (0.891) | 1.116 (1.874) | 0.872 | 0.111 | 0.027 | 0.946 |
| SER | 1.00 | 4.00 (6.459) | 0.770 (0.534) | 1.418 (0.374) | 0.889 | 0.095 | −0.071 | 0.941 |
| TAE | 0.50 | 2.999 (3.041) | 0.994 (0.253) | 2.299 (1.182) | 0.989 | 0.033 | 0.058 | 0.994 |
| VIN | 0.70 | 2.557 (305.636) | 1.204 (41.006) | 2.173 (89.386) | 0.792 | 0.154 | 0.277 | 0.871 |

[1] Estimates of parameters of Equation (6); [2] adjusted $R^2$, root mean square error (RMSE), coefficient of residual mass (CRM), concordance correlation coefficient (CCC).

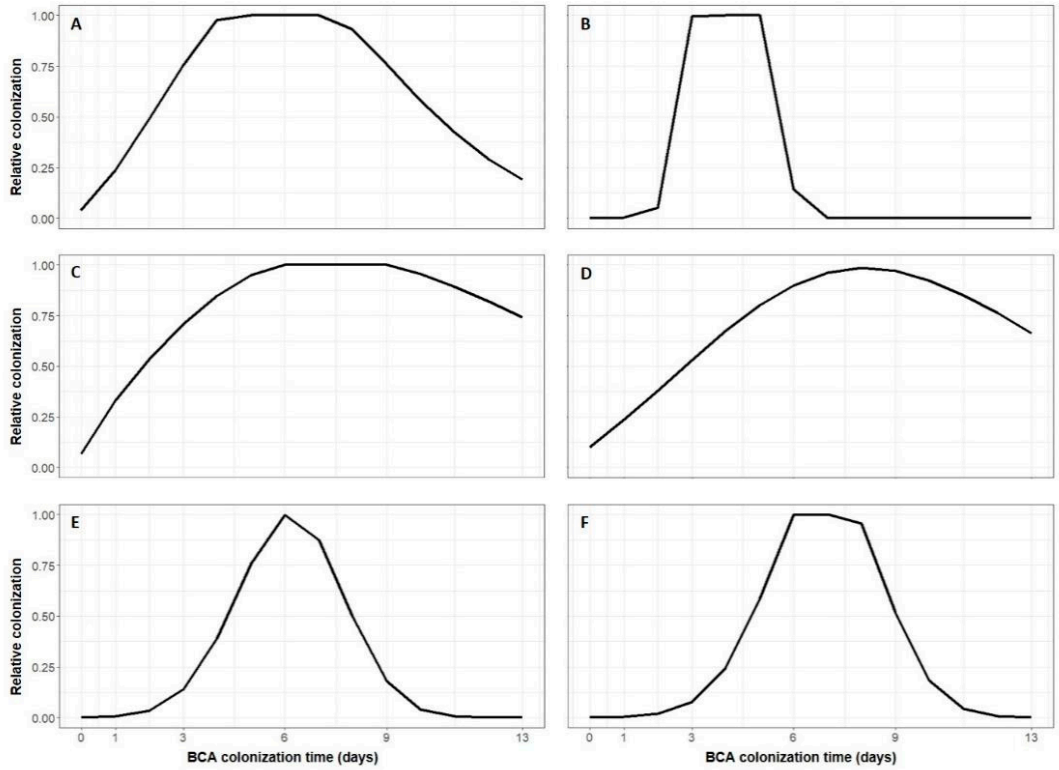


**Figure 6.** Progress of the relative colonization of bunch trash that was treated with one of six BCAs (**A**: AMY; **B**: BOT; **C**: NOL; **D**: SER; **E**: TAE; **F**: VIN, see Table 1) and then incubated at the optimal temperature (AMY: 30 °C, BOT: 25 °C, NOL: 15 °C, SER: 25 °C, TAE: 25 °C, and VIN: 30 °C) and 100% RH; after 1, 3, 6, 9, and 13 days, the number of CFUs was assessed. Lines were drawn using Equation (6) and the parameters listed in Table 4.

## 4. Discussion

Grapevine bunch trash colonized by *B. cinerea* has been considered an important source of inoculum for infection from flowering to ripening [46–49] and has been related to the severity of BBR at harvest [50–52]. Thus, the reduction in *B. cinerea* inoculum on bunch trash from flowering to pre-bunch closure may contribute to BBR control, as shown in a meta-analysis of 116 studies [53,54] and in several field experiments [12,13,55].

To understand how to reduce this *B. cinerea* inoculum, researchers have studied the effects of removing the bunch trash from clusters at fruit set by the use of compressed air or leaf blowers [56], or by the application of fungicides [52] between the end of flowering (A) and pre-bunch closure (B); pre-bunch closure is considered the last stage for disinfesting the bunch trash inside the cluster because subsequent expansion of berries impedes the deposition of fungicides on the bunch trash [52,57]. BCAs have also been studied as alternatives for reducing the colonization of bunch trash by *B. cinerea* [12,13,58]. In a previous report [13], the saprophytic colonization of bunch trash by *B. cinerea* and its consequent sporulation were reduced by 36% to 93% by the application of BCAs at flowering. This high variability in BCA efficacy may be related to the influence of environmental conditions on BCA survival, establishment, and growth [26,28,59]. The latter possibility was supported by a recent simulation study, which found that—considering the combined effects of: (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—the environmental conditions are the most important factor influencing biocontrol efficacy, accounting for >90% of the variation in model simulations [21]. Environmental conditions at the time of BCA application and in the following days could therefore explain much of the inconsistency in BCA efficacy often observed in field applications [13–16].

The results of the current study confirmed that environmental conditions greatly affect the colonization of bunch trash by biocontrol microorganisms. The current study also developed equations that predict the colonization dynamics of bunch trash by BCAs as affected by temperature and humidity; to our knowledge, no similar equations have been previously developed for bunch trash.

The study was conducted under controlled environmental conditions in growth chambers rather than in the field, and several previous studies on biocontrol microorganisms have also been conducted under controlled environmental conditions [15,60–64]. The advantages of using controlled environmental conditions are obvious in that they enable the assessment of specific T/RH regimes. That equations developed under controlled environments may correctly predict fungal growth under field conditions, where temperature and humidity fluctuate and interact with other factors, such as solar radiation or wind, has been demonstrated for several fungi [65–67]. Whether the equations developed under controlled conditions in the current study correctly predict BCA colonization of bunch trash in the field remains to be determined.

The results of this study also showed that optimal T/RH conditions for bunch trash colonization differed among the BCAs, with some growing better at T > 25 °C and RH > 80% (AMY and VIN), others at 20 < T < 25 °C and RH > 90% (BOT, SER, and TAE), and one at T < 20–25 °C and 80 to 90% RH (NOL). The observed T/RH patterns did not depend on whether the microorganism is a fungus, yeast, or bacterium, or whether the BCA contains different species of *Bacillus* or different strains of the same *Bacillus* species (SER containing *B. subtilis*, AMY and TAE containing *B. amyloliquefaciens* D747 and FZB24, respectively). That the response to the environment varies among taxa, species, and strains was expected, as was the commercial formulation influencing the fitness of BCAs [21].

This variability in the response of commercial BCAs to T/RH could facilitate *B. cinerea* control, because it would enable growers to select the best BCA to be used for a specific application in the vineyard based on the weather conditions at the time of application and weather forecasts in the next days [30]. For example, in moist and cold environments, a BCA able to colonize bunch trash at high RH levels and low temperatures should be preferred because it would have a higher probability of growing and competing with *B. cinerea* on the bunch trash. Although the efficacy

of BCAs in reducing the sporulation of *B. cinerea* on bunch trash was not assessed in the present study, colonization by biocontrol microorganisms has been considered a prerequisite for successful biocontrol [64,68], and colonization of plant tissue by BCAs was assumed to directly affect biocontrol efficacy [25]. In this work, the colonization of bunch trash over time varied among BCAs, with some of them growing and declining faster than others (Figure 6). These dynamics may depend on (i) the chemical composition of the bunch trash, (ii) the nutritional requirements of each microorganism, and (iii) the rates at which nutritional sources in the bunch trash are depleted over time [26]. To grow and reproduce, microorganisms require a carbon (C) source, a nitrogen (N) source, vitamins, minerals, and other nutrients, and these requirements can be quite specific [69]. Previous research revealed that the suitability of C sources, N sources, C concentrations, and C/N ratios for mycelial growth and sporulation substantially differed among several biocontrol fungi [70] and also differed among fungal growth stages [69]. The BCAs used in the current research were different taxa and probably differ in their nutritional requirements, which may help explain the different dynamics in Figure 6. It follows that research is needed on the nutritional requirements of biocontrol microorganisms in relation to their growth stage (e.g., growth vs. reproduction and growth vs. synthesis of secondary metabolites) and the target plant substrate (e.g., bunch trash vs. ripening berries). Concerning the plant substrate, for example, BOT required fewer than 5 days for maximal colonization of bunch trash at 25 °C, but required more than 10 days at the same temperature for maximal efficacy on berries [30].

The differences among BCAs with respect to BCA colonization time under optimal T/RH conditions (Figure 6) can be useful for timing their application in order to control BBR. At 13 days after BCA application (i.e., the last time that the BCA populations were assessed in this work), colonization rapidly declined for some BCAs (especially BOT) but remained high for others (NOL and SER). Rapid population declines after field applications of BCAs have been previously observed [71,72], even though the biocontrol efficacy remained high in some studies [12,13,16]. Therefore, the minimal level of BCA colonization required to prevent *B. cinerea* colonization of bunch trash should be further investigated to predict how long an early season BCA application remains effective and then determine whether and when a second BCA application may be needed.

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