Quantification of *Botrytis cinerea* in Grapevine Bunch Trash by Real-time PCR

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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.

Keywords: Botrytis bunch rot, grey mold, bunch trash, hydrolysis probe-based qPCR assay, colonization coefficient, colonization rate, sporulation potential.

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; Ciliberti et al. 2016; Ciliberti et al. 2015b; 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 *B. 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 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 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 are as follows: Bc3 F: 5′-GCT GTA ATT TCA ATG TGC AGA ATC C-3′; Bc3 R: 5′-GGA GCA ACA ATT AAT CGC ATT TC-3′; Bc3 P: 5′-6-FAM-TCA CCT TGC AAT GAG TGG-BHQ-1-3′; Res F: 5’-CGA GGA ATT TAG AAA CGC TCA AC-3’; Res R: 5’-GCT GTG CCA ATG GCT AGG A-3’; and Res P: 5’-JOE-TGC CAA GGG TCC GGC CAC C-TAMRA-3’.

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 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 1250 conidia/µl) or fresh

mycelium (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:

$$CC = DNA_{B.cinerea} / DNA_{V.vinifera} + \Delta CC.$$

DNA amounts were obtained by transforming the Cq values of both targets (*B. cinerea* and *V. vinifera*) according to the standard curves obtained from the serial dilution assays (Table 2): DNA (ng/µl) = $10^{[(Cq\ value - y\text{-}axis\ intercept)/slope]}$. 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 asl). The CA vineyard was planted to cv. Merlot, which is highly susceptible to *B. cinerea* (Bisiach et al. 1996; Corvi and Tullio 1979). 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 flower (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 2 h. 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 (Mipaaf, 2017). Between-rows was grass, with vines pruned to 10-12 buds per cane, and emergency irrigation. The CO vineyard followed a conventional pest management strategy. Between-rows was grass, with vines

long pruned and irrigated. 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 1979).

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 flower (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 berries with latent infection (ILI) 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 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 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 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).

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. 1A) and to the quantity of mycelium of *B. cinerea* added to the bunch trash (Fig. 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. 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. 2B and 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 these variables (R^2 = 0.92 and 0.97, respectively).

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. 3A), the SP (sporulation potential) (Fig. 3B), and the ILI (incidence of latent infection on berries) (Fig. 3C) (P<0.001 for all three variables; Table 3). Year had a significant effect on CC (P ≤0.001): 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. 4A), < 3 (Fig. 4B), or < 5 (Fig. 4C).

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. 2018). 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.*

ACKNOWLEDGMENTS

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506 TABLE 1. List of isolates screened during specificity tests of the real-time qPCR assay used to
507 quantify *B. cinerea* in grape bunch trash

508

Genus and species	Isolate code	qPCR result[b]
Alternaria alternata	5	-
Alternaria sp.	23	-
Aspergillus flavus	4	-
Aspergillus niger	A1	-
Botrytis cinerea	213T and 351V	+
Erysiphe necator	FP[a] 2017 and FP 2018	-
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] FP: Field population and year of collection.

[b] '+' indicates amplified, and '-' indicates not amplified.

509

TABLE 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.

TABLE 3. Results of the ANOVA performed to explore main effects of fungicide treatment, vineyard and year, and main effect interactions, on the quantity of DNA of *B. cinerea* in bunch trash (defined as the colonization coefficient determined by the qPCR assay), the sporulation potential of bunch trash (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 (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/l of water, or not treated, with the experiment conducted in 2016 and 2017 in three vineyards in Italy.

Main effects and interactions	d.f.[a]	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] degrees of freedom

532 **Figure captions**

533

534 **Fig. 1.** Relationship between the colonization coefficient (the ratio between DNA concentrations of
535 *Botrytis cinerea* and *Vitis vinifera* as determined by the qPCR assay) and (A) the number of conidia
536 added per gram of bunch trash, and (B) the weight of fresh mycelium added to the bunch trash in
537 inoculation experiments. Markers indicate means, whiskers indicate standard errors, and dotted lines
538 indicate the linear relationships; in A: Y=*0.011+0.001*X (R^2=0.92); in B: Y=*1.75+710.74*X
539 (R^2=0.97).

540

541

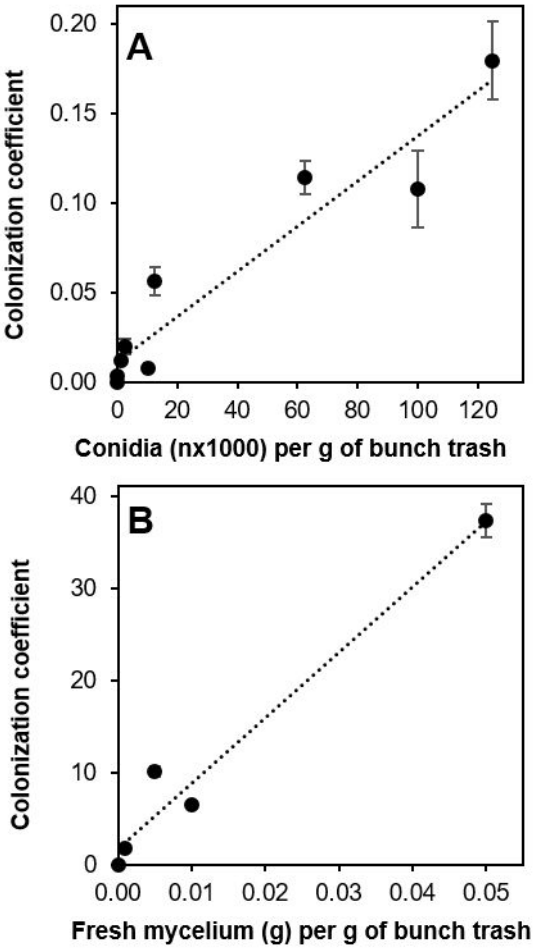
542 **Fig. 2.** Relationship between the colonization coefficient (the ratio between DNA concentrations of
543 *Botrytis cinerea* and *Vitis vinifera* as determined by the qPCR assay) of grape bunch trash and (A)
544 the proportion (%) of bunch trash that had been inoculated with and colonized by *B. cinerea*, (B) the
545 sporulation potential of the bunch trash (expressed as the number of conidia produced per gram of
546 bunch trash as determined by incubating the bunch trash in humid chambers), and (C) bunch trash
547 colonization (expressed as the proportion (%) of bunch trash pieces that were colonized by *B. cinerea*
548 as determined by plating the bunch trash on PDA). The grape bunch trash was inoculated with *B.*
549 *cinerea* prior to being assayed for DNA of *B. cinerea*, sporulation potential, and colonization rate.
550 Markers indicate means, whiskers indicate standard errors, and dotted lines indicate linear and non-
551 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);
552 in C: Y=1.17E+07/(1+exp(*4.31-1.41*X)) (R^2=0.99).

553

554

Fig. 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 ($P<0.001$).

Fig. 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 ($P=0.6$).



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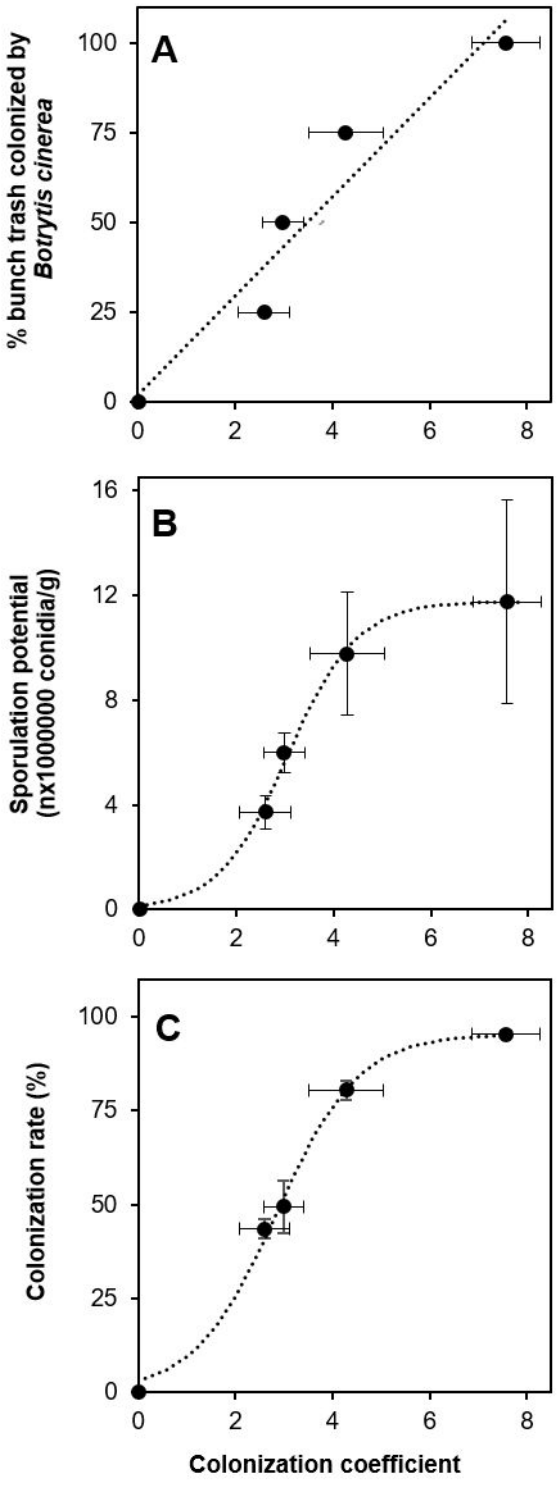

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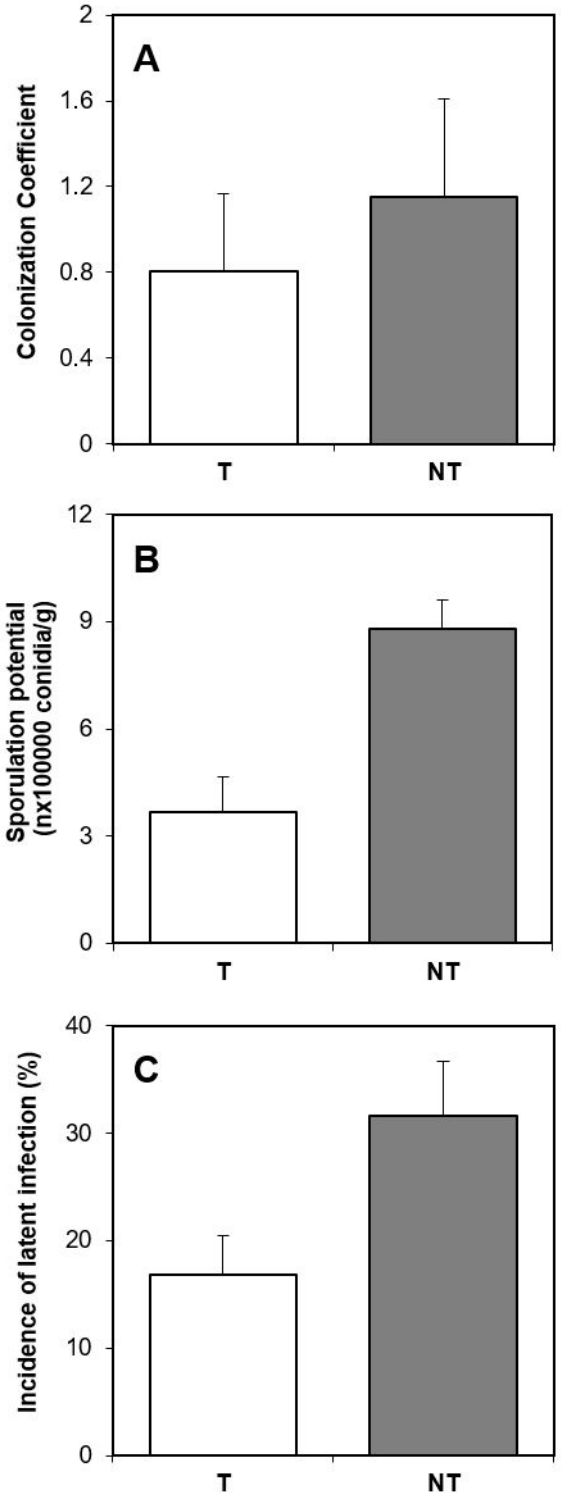

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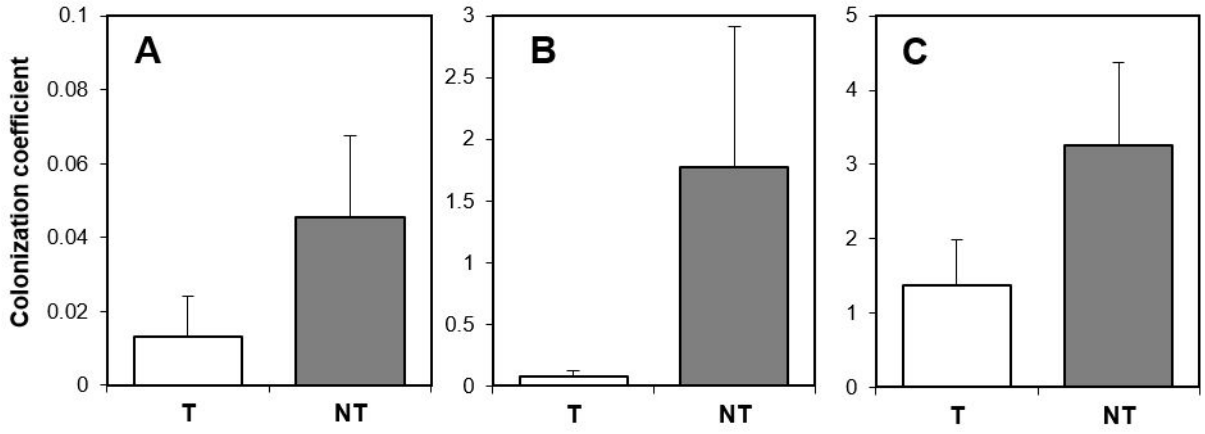

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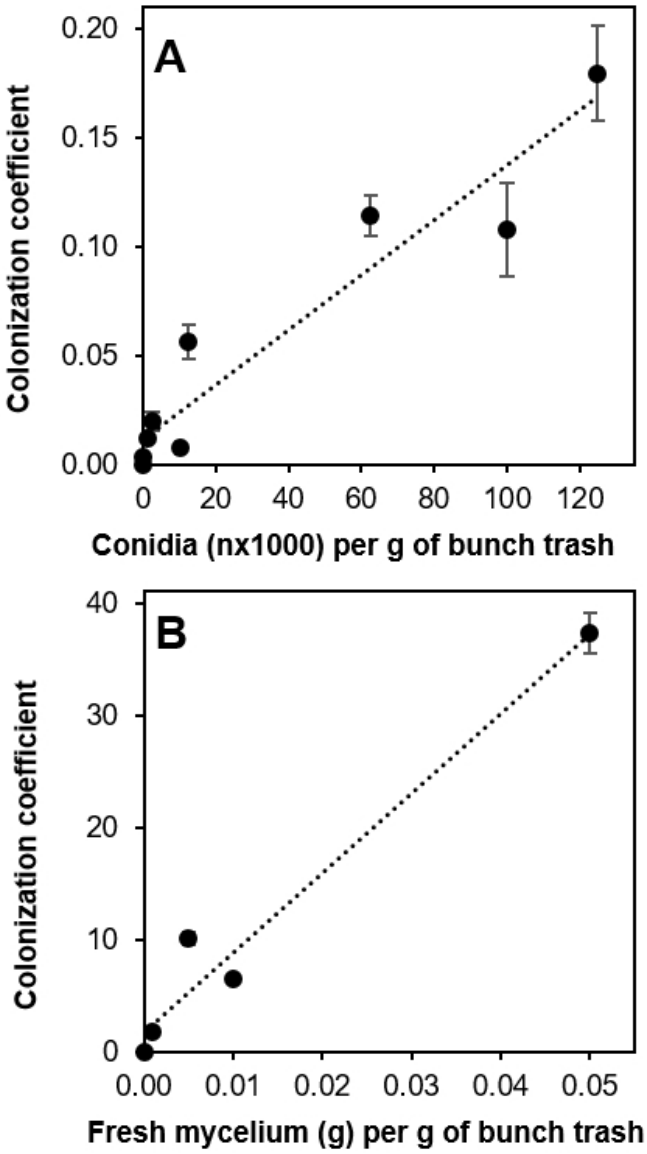

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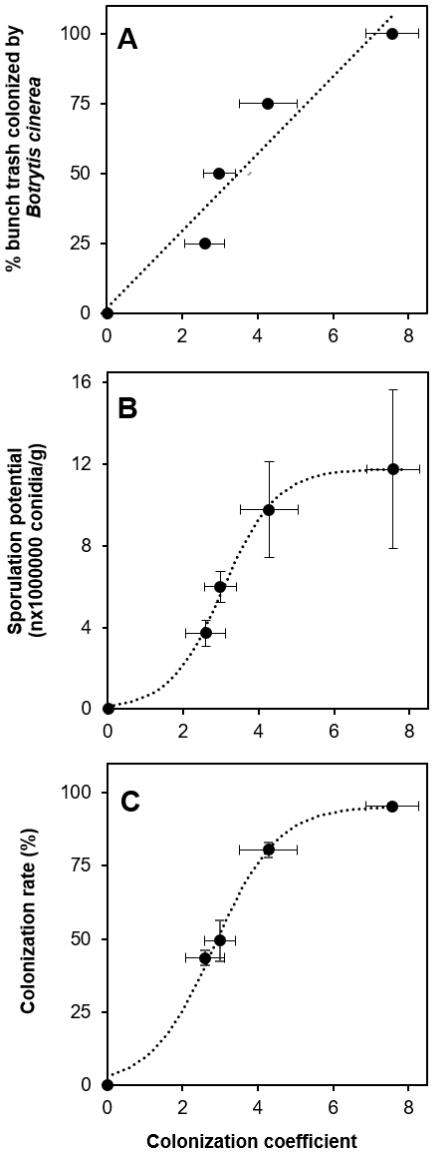

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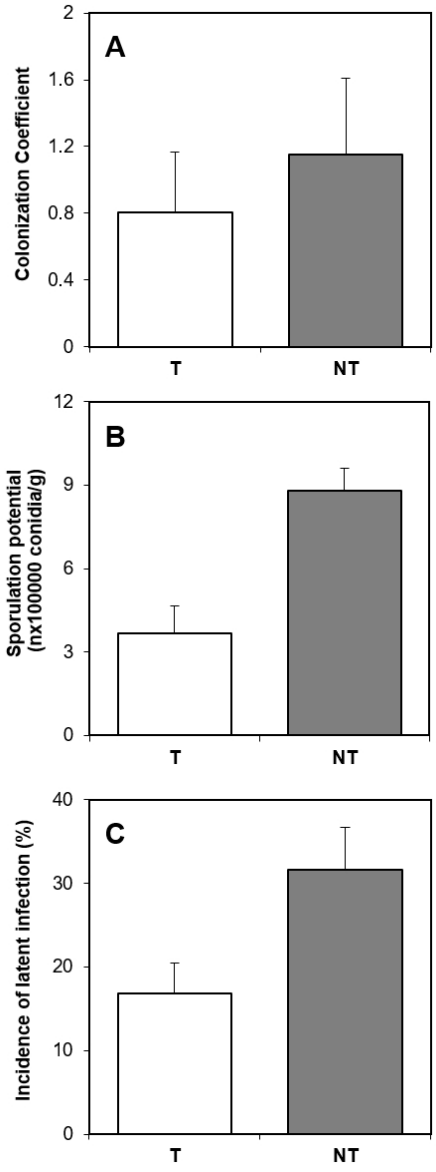

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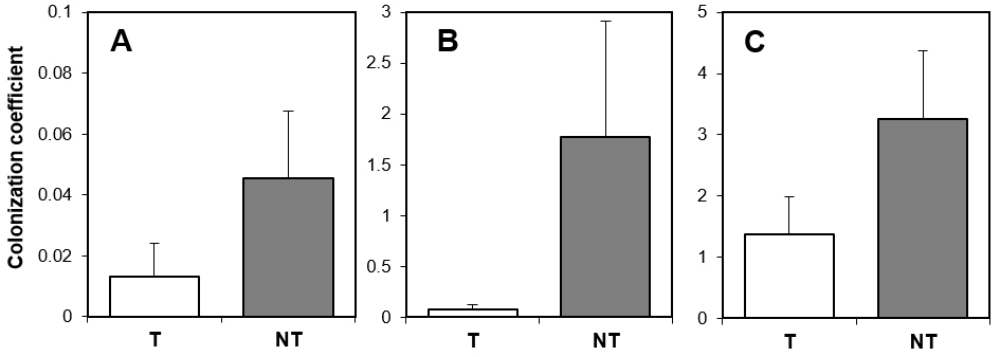

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160x58mm (150 x 150 DPI)