Research Article

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Suppression of *Botrytis cinerea* on necrotic grapevine tissues by early-season applications of natural products and biological control agents

Carlos Calvo-Garrido,[a] Inmaculada Viñas,[a] Philip AG Elmer,[b] Josep Usall[c] and Neus Teixidó[c]*

Abstract

BACKGROUND: Necrotic tissues within grape (*Vitis vinifera*) bunches represent an important source of *Botrytis cinerea* inoculum for *Botrytis* bunch rot (BBR) at harvest in vineyards. This research quantified the incidence of *B. cinerea* on necrotic floral and fruit tissues and the efficacy of biologically based treatments for suppression of *B. cinerea* secondary inoculum within developing bunches.

RESULTS: At veraison (2009 and 2010), samples of aborted flowers, aborted fruits and calyptras were collected, and the incidence and sporulation of *B. cinerea* were determined. Aborted fruits presented significantly higher incidence in untreated samples. Early-season applications of *Candida sake* plus Fungicover®, Fungicover alone or *Ulocladium oudemansii* significantly reduced *B. cinerea* incidence on aborted flowers and calyptras by 46–85%. Chitosan treatment significantly reduced *B. cinerea* incidence on calyptras. None of the treatments reduced *B. cinerea* incidence on aborted fruits. Treatments significantly reduced sporulation severity by 48% or more.

CONCLUSIONS: Treatments were effective at reducing *B. cinerea* secondary inoculum on necrotic tissues, in spite of the variable control on aborted fruits. This is the first report to quantify *B. cinerea* on several tissues of bunch trash and to describe the effective suppression of saprophytic *B. cinerea* inoculum by biologically based treatments.

Keywords: biological control; *Candida sake* CPA-1; chitosan; floral debris; *Ulocladium oudemansii* HRU3; *Vitis vinifera*

1 INTRODUCTION

Several infection pathways have been described for *Botrytis cinerea*, the causal agent of *Botrytis* bunch rot (BBR) of wine grapes, and these were summarised by Elmer and Michailides.[1] It is generally acknowledged that early-season infection by *B. cinerea* of senescent and necrotic floral grape tissues during flowering and pre-bunch closure determine bunch rot at harvest,[2–4] while late-season infections of intact mature berries are associated with both conidial and mycelial inoculum sources from infection of the style, stamens, calyptra, aborted flowers and aborted berries.[1] Other necrotic tissues have been identified as potential inoculum sources within developing bunches and include leaf fragments or tendrils,[5,6] or the ring of necrotic tissue at the pedicel–receptacle complex ('cap scar'),[2,7] whereas tissues trapped within the ripening bunch have been considered important sources of *B. cinerea* for late-season development of BBR, especially when bunches are compact.[8] In addition, other epidemiological studies have also shown the high relative importance of treatments early in the season, which interfere with the pathways described above, for effective BBR control at harvest.[2,9]

This knowledge of the importance of early-season infections has provided opportunities for more targeted chemical control.[10,11] However, the application of synthetic fungicides in vineyards worldwide has become less popular owing to the ease with which *B. cinerea* populations quickly adapt to new fungicide chemistry[12–14] and reports of harmful environmental and human health risks associated with some synthetic fungicides.[15] Constraints associated with fungicide use and the increasing number of organic growers highlight the need for alternative or complementary control strategies against BBR.

In organic viticulture, synthetic botryticides are not permitted, and the number of alternative products available for BBR control is reduced to the application of salts, essential oils, compost and plant extracts.[16,17] Moreover, results are sometimes variable at

* *Correspondence to: Neus Teixidó, IRTA, XaRTA-Postharvest, 191 Rovira Roure, 25198 Lleida, Catalonia, Spain. E-mail: neus.teixido@irta.cat*

a *Food Technology Department, Lleida University, XaRTA-Postharvest, Agrotecnio Center, Lleida, Catalonia, Spain*

b *The New Zealand Institute for Plant and Food Research Limited, Ruakura Research Centre, Hamilton, New Zealand*

c *IRTA, XaRTA-Postharvest, Lleida, Catalonia, Spain*

harvest, and specific early-season treatments are very limited. In response, several research groups worldwide have investigated alternative, organically acceptable treatments such as those based upon biological control agents (BCAs), natural products (NPs) and biologically based elicitors of host defence for *Botrytis* control in viticulture. The mode of action of these strategies is usually preventive, and the curative effect is generally reduced. Thus, applications early in the season are highly recommended in order to achieve a significant control of secondary inoculum and hence prevent BBR epidemic outbreak, especially when dealing with alternative treatments.

In New Zealand, an isolate of *Ulocladium oudemansii* (HRU3) consistently reduced BBR in grapes, while saprophytic colonisation of senescent and necrotic calyptras and aborted fruitlets early in the growing season was identified as its primary mode of action.[16,18,19] This isolate was successfully commercialised and is the active ingredient in BOTRY-Zen®, a BCA product that is approved for early-season *Botrytis* suppression in organic and conventional viticulture in New Zealand. Suppression of BBR development was when this BCA was integrated with mid- and late-season commercialised natural products, including chitosan.[19,20]

Chitosan is a natural carbohydrate polymer extracted from crustaceans, and several studies report the efficacy of different chitosan fractions against several phytopathogenic fungi,[21,22] including *B. cinerea* on grapes.[23–25]

The CPA-1 strain of *Candida sake* was isolated, optimised and formulated by the Postharvest Pathology research group at IRTA, Lleida. When it was field applied with the additive Fungicover® to wine grapes in conventional and organic vineyards in Catalonia, Spain, BBR at harvest was significantly reduced.[24,26] Fungicover is a natural product consisting of an emulsion of fatty acids and polysaccharides in aqueous–alcoholic solution, and it is principally used by researchers as a coating and protective agent for *C. sake* field applications able to improve BCA survival.[26]

The biologically based products described above have shown *B. cinerea* control under a variety of conditions. However, the ability of *U. oudemansii* to suppress *B. cinerea* on necrotic tissues in hot, dry winegrowing regions has not been reported, and there are no published data on the effect of chitosan or *C. sake* applications on *B. cinerea* development in necrotic bunch debris.

The objectives of this study were (a) to quantify the relative incidence of *B. cinerea* infections on different necrotic tissues within bunches of organic wine grapes 'Macabeu' and (b) to determine the efficacy of early-season applications of three different biologically based treatments in terms of their ability to reduce *B. cinerea* inoculum.

2 MATERIALS AND METHODS

2.1 Vineyard field trials

Field trials were established in two commercial vineyards (identified here as OLV and CTD) and were carried out over two growing seasons. The vineyards were 10 km apart and located in a traditional winemaking area (designation of origin Costers del Segre, Lleida, Catalonia, Spain). This region typically experiences cold winters and hot, dry summers (mean annual precipitation 428.4 mm, mean average temperature 13.9 °C).[27] The winemaking variety selected was 'Macabeu', a white variety characterised by a large, compact bunch structure that is highly susceptible to BBR. The vines in OLV and CTD were trained to a goblet system and were planted 2.5 m apart between the rows and 2 m apart within each row.

The OLV vineyard was certified organic by the Catalan Committee for Organic Agriculture Production (CCPAE) and was sprayed to control plant diseases other than BBR. In 2009, the vineyard received three applications of 99% sulphur (w/w) prior to flowering, one more application of 99% sulphur (w/w) plus 98% silicon (w/w) at pre-bunch closure and finally one application of *Bacillus thuringiensis* var. Kurstaki at veraison. In 2010, treatments included one application of 99% sulphur (w/w) prior to flowering, two applications of 60% sulphur (w/w) plus 4% copper oxychloride (w/w) at pre-bunch closure, one application of 38% copper oxychloride (w/w) at pre-bunch closure and two applications of *B. thuringiensis* var. Kurstaki at veraison. The CTD vineyard was conventionally managed and received one 80% sulphur spray (w/w) at pre-bunch closure, but no antibotrytic fungicide applications. All products in both fields were applied at the doses and application rates recommended by the product manufacturers.

Two investigations were carried out in 2009 and 2010. In the first study, a range of necrotic tissues were sampled from untreated grape bunches in order to quantify the incidence and sporulation potential of *B. cinerea* infections in necrotic bunch trash tissues of the white wine variety Macabeu. In the second study, necrotic tissues were sampled from developing grape bunches at veraison in order to determine the effect of biologically based treatments on *B. cinerea* development on treated necrotic bunch trash debris. In the first study, OLV and CTD vineyards were sampled in 2009, whereas in 2010 only the OLV vineyard was used. The second study was carried out in the OLV vineyard in 2009 and 2010.

2.2 Part 1: Source of *B. cinerea* on necrotic tissues in developing bunches of Macabeu

At veraison, samples of necrotic tissues that were trapped within the developing bunches were taken from four bunches per replicate. Bunches were randomly selected from the sample vines, and in the laboratory each bunch was divided into four sections (top left, top right, middle and bottom of the bunch). Three different samples of necrotic tissues were collected with sterile tweezers from each bunch section, consisting of one aborted flower, one calyptra and one aborted fruit. Aborted fruits were considered to be different from aborted flowers when the swollen ovary of the transforming flower presented a diameter greater than 2 mm. Trays, gloves and tweezers were surface sterilised with ethanol prior to sectioning of each bunch and removal of necrotic samples.

The four samples of each tissue type from each bunch were placed on a sterile petri dish with Whatman No. 1 (85 mm diameter) filter paper that had been moistened with 1.25 mL of sterile distilled water to create a high-humidity chamber for *B. cinerea* development. The four high-humidity chambers corresponding to four bunches constituted one replicate. In total there were 16 aborted flowers, calyptras and aborted fruit per replicate. The petri dishes were then incubated at 20 °C in the dark for 10 days prior to assessment.

The sampling protocol described in this section was the same for both experiments. In the first study, samples were taken from untreated plots only, while, in the second study, samples were taken both from plots that had received biologically based treatments and from untreated plots.

2.3 Part 2: Effect of biologically based treatments on *B. cinerea* colonisation of necrotic floral tissues

2.3.1 Microbial antagonists and natural products

The BCA *C. sake* CPA-1 was originally isolated from apples by the University of Lleida IRTA centre and was deposited at the Coleccion Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. Stock cultures were stored on nutrient yeast dextrose agar (NYDA) medium (nutrient broth, 8 g L^{-1}; dextrose 10 g L^{-1}; agar 15 g L^{-1}) at 4 °C. When required, *C. sake* CPA-1 was subcultured onto NYDA plates at 25 °C. Then, subcultured cells suspended on potassium phosphate buffer (KH_2PO_4 0.2 M, 70 mL; K_2HPO_4 0.2 M, 30 mL; deionised water 300 mL) were added as inoculum starter to 5 L of molasses-based (MB) medium (cane molasses 40 g L^{-1}; urea 1.2 g L^{-1}; water activity $a_w = 0.996$), with adjustment of the initial concentration to 1×10^6 CFU mL^{-1}. Cell pellets were obtained by centrifugation at 6831 × *g* for 10 min at 10 °C after 40 h of liquid fermentation at 25 °C, 400 rpm agitation speed and 150 L h^{-1} aeration level. Resuspended pellets were then formulated in an isotonic solution, with adjustment of the water potential with trehalose as described previously [28] Prior to field application, the BCA additive Fungicover (Biodúrcal S.L., Granada, Spain) was added to the *C. sake* suspension (50 g L^{-1}) and then mixed with the aid of a handheld paint mixer to ensure thorough mixing. Fungicover was included because this additive has been shown to aid field survival of *C. sake*.[26]

The BCA *U. oudemansii* was obtained as a dry, water-dispersible granule (BOTRY-Zen®, AI *U. oudemansii* 2.5×10^8 CFU L^{-1}; Botry-Zen 2010 Ltd, Dunedin, New Zealand). Chitosan was sourced from ARMOUR-Zen® as a liquid concentrate (AI chitosan 1.44 g L^{-1}; Botry-Zen 2010 Ltd, Dunedin, New Zealand). Because the water-dispersible granule and liquid concentrate formulations did not contain wetting agents, final solutions for field trials were prepared in water containing 0.5 mL L^{-1} of the wetting agent Mojante Inagra (alquil poliglicol 20% w/v; Sipcam Inagra S.A., Valencia, Spain) to improve tissue wetting. For the field application of chitosan treatment, 1.05 g L^{-1} of sodium bicarbonate was added to the final solution to adjust to pH = 7, as it can improve *B. cinerea* suppression by chitosan (Reglinski T, private communication).

2.3.2 Experimental design

In 2009, six early-season treatments were evaluated for their ability to reduce *B. cinerea* infection of necrotic bunch tissues. The treatments and timing of applications during the growing season are summarised in Table 1. In 2010, treatments consisted of two applications of *C. sake* CPA-1 and Fungicover at 80% flowering and pre-bunch closure (Table 1).

The 2009 and 2010 experiments, including biologically based treatments, were carried out in the OLV field, with four replicate plots containing seven vines for each treatment laid out in a randomised block design. All treatments in both seasons were applied with a motorised backpack sprayer (WJR2225 model; Honda Motor Company Ltd, Germany) at 1.5×10^6 Pa pressure and with a 1 mm nozzle focusing on grape bunches to the point of run-off.

After treatment applications, necrotic tissue samples from treated and untreated plots were collected and processed at veraison, as previously described for the first study evaluating inoculum sources.

Table 1. Summary of treatments applied early in the growing season for *B. cinerea* suppression in an organic vineyard cv. Macabeu in 2009 and 2010

Treatment[a]	1–5% flowering	80% flowering	Pre-bunch closure
2009			
Control	—	—	—
Chitosan	Chitosan	Chitosan	Chitosan
U. oudemansii	*U. oudemansii*	*U. oudemansii*	*U. oudemansii*
CS + FC	CS + FC	CS + FC	CS + FC
CS low + FC	CS low + FC	CS low + FC	CS low + FC
FC	FC	FC	FC
2010			
Control	—	—	—
CS + FC	—	CS + FC	CS + FC
FC	—	FC	FC

[a] Control: untreated; Chitosan: chitosan 1.44 g L^{-1} + wetting agent 0.5 mL L^{-1} + 1.05 g L^{-1} of $NaHCO_3$; *U. oudemansii*: *U. oudemansii* 2.5×10^6 CFU mL^{-1} + wetting agent 0.5 mL L^{-1}; CS: *C. sake* 5×10^7 CFU mL^{-1}; CS low: *C. sake* 1×10^7 CFU mL^{-1}; FC: Fungicover 50 g L^{-1}. Treatments consisted of three applications. Applications were carried out over four replicates of each treatment in a randomised block design.

2.4 Colonisation assessment of *B. cinerea* and *U. oudemansii* in necrotic bunch trash tissues

In both the first and the second studies in 2009 and 2010, each necrotic tissue sample was visually assessed using a stereomicroscope after 10 days of incubation in high-humidity chambers. The sporulation severity of *B. cinerea* was measured using a sporulation index with a 0–5 scale, where 0 = no visible conidiophores, 1 = 1–5, 2 = 6–10, 3 = 11–20, 4 = 21–40 and 5 = 40–100 conidiophores. In samples treated with *U. oudemansii* in 2009, the area of necrotic tissues covered in sporulating *U. oudemansii* was determined using a 0–100% scale, where no *U. oudemansii* visible = 0%, trace quantities visible = 1–5%, low–moderate colonisation = 6–25%, moderate colonisation = 26–50%, moderate–high colonisation = 51–75% and high colonisation = 76–100%. The mean percentage value of *U. oudemansii* colonisation was calculated for each class in the scale (0, 3, 15.5, 38, 63 and 88% respectively) and then multiplied by the number of samples in each class eventually to obtain the average percentage coverage per replicate tissue type.

To calculate the mean values of *B. cinerea* incidence, *B. cinerea* sporulation severity, *U. oudemansii* incidence and percentage tissue area colonised by *U. oudemansii*, use was made of data from visual assessment of the 16 necrotic samples of each tissue type per replicate.

2.5 *Botrytis* bunch rot assessment

BBR at harvest in the untreated plots used in the bunch trash studies was measured in the 2009 and 2010 seasons at the field sites by assessing 50 bunches per replicate plot (25 bunches from each side of the plot). BBR incidence (number of bunches with *B. cinerea*) was expressed as a percentage, and BBR severity was visually estimated as the percentage of the bunch infected.

2.6 Meteorological data

Hourly measurements of temperature (*T*), relative humidity (RH), rainfall (RN) and leaf wetness (LW) were collected using a weather

station (Decagon Services Inc., Pullman, WA) placed beside one of the experimental plots in the two seasons of the study.

2.7 Statistical analysis

Analysis of variance was performed using JMP8 (SAS Institute Inc., Cary, NC) for all datasets. Significant treatment differences were determined using Student's LSD *t*-test ($P = 0.05$) for the effect of biologically based treatments on *B. cinerea*. Tukey's test ($P = 0.05$) was used to separate necrotic tissue means for significant differences. Sporulation index data of *B. cinerea* samples were transformed [$\sqrt{(x + 0.5)}$] prior to ANOVA to improve homogeneity of variances.

3 RESULTS

3.1 Source of *B. cinerea* on necrotic tissues in developing bunches of Macabeu

B. cinerea was detected on all necrotic tissue types sampled from untreated plots at both sites (CTD and OLV in 2009) and in both years (OLV in 2009 and 2010) (Fig. 1). No significant interactions ($P < 0.05$) were detected between years, field sites or *B. cinerea* incidence (data not shown). Therefore, incidence data in the different tissue types for 2009 and 2010 and the two field sites could be pooled for the statistical analysis.

Overall, the incidence of *B. cinerea* in aborted fruits of Macabeu (38%) was significantly higher ($P < 0.05$) than the incidence in aborted flowers or calyptras (18% and 24% respectively) (Fig. 1).

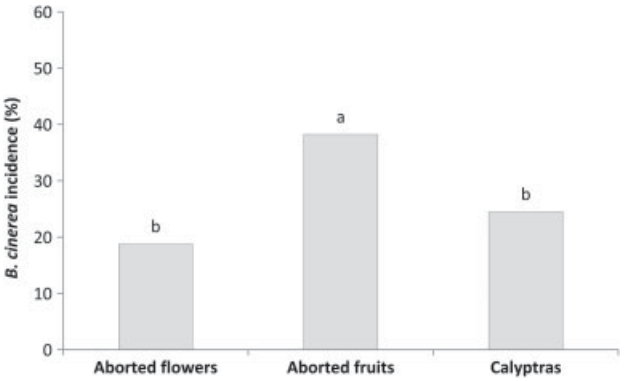

Figure 1. Incidence of *B. cinerea* natural infections in necrotic tissues sampled from within immature grape bunches from untreated plots. Data are average of two years and two sites (OLV field in 2009 and 2010; CTD field in 2009). Values are the means of four replicate plots. Mean values with the same letter are not significantly different ($P = 0.05$) according to Tukey's test.

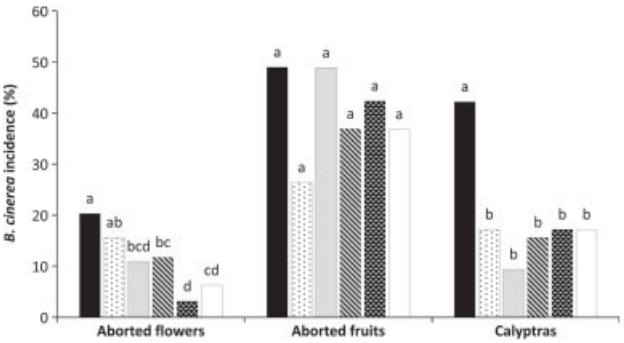

Figure 2. Effect of early-season biologically based treatments on *B. cinerea* incidence in necrotic tissues from grape bunches of Macabeu sampled at 2009 veraison in the OLV organic vineyard. Control (■): untreated; Chitosan (□): chitosan 1.44 g L^{-1} + wetting agent 0.5 mL L^{-1} + 1.05 g L^{-1} of $NaHCO_3$; *U. oudemansii* (▤): *U. oudemansii* 2.5 × 10^6 CFU mL^{-1} + wetting agent 0.5 mL L^{-1}; CS + FC (▧): *C. sake* 5 × 10^7 CFU mL^{-1} + Fungicover 50 g L^{-1}; CS low + FC (▩): *C. sake* 1 × 10^7 CFU mL^{-1}; FC (▥): Fungicover 50 g L^{-1}. Treatments were applied to vineyard plots at 1–5% flowering, 80% flowering and pre-bunch closure. Values are the mean of four replicate plots. Mean values with the same letter are not significantly different ($P = 0.05$) according to LSD Student's t-test.

3.2 Reduction of *B. cinerea* incidence and sporulation in necrotic tissues by early-season biologically based treatments – 2009 field studies

The incidence of *B. cinerea* on untreated necrotic tissues in 2009 compared with tissues that had been field sprayed with biologically based treatments is summarised in Fig. 2. In the untreated plots the incidence of *B. cinerea* ranged from 20% in aborted flowers to 42 and 48% in calyptras and aborted fruits respectively. All treatments significantly reduced ($P < 0.05$) *B. cinerea* incidence in the necrotic calyptras, ranging from 59% (Chitosan, CS low + FC, FC) to 77% (*U. oudemansii*). All treatments, with the exception of chitosan, significantly reduced *B. cinerea* incidence in the aborted flowers ($P < 0.05$) by 46% (*U. oudemansii*) to 84% (CS low + FC) compared with the untreated control. Overall, there were no significant differences ($P < 0.05$) in *B. cinerea* incidence among the effective treatments for any tissue type, with the exception of CS + FC and CS low + FC in the aborted flowers. Three applications of the biologically based treatments did not significantly reduce *B. cinerea* incidence ($P < 0.05$) in the aborted fruits.

The mean number of *B. cinerea* conidiophores across all tissue types was expressed using a sporulation index and was 0.97 from samples taken from the untreated plots (Fig. 3). This value equates to an average of 1–5 conidiophores per tissue sample. All biologically based treatments significantly reduced the *B. cinerea* sporulation index value ($P < 0.05$) by 50% or more compared with untreated samples (Fig. 3). There were no significant differences between the treatments ($P < 0.05$), and the sporulation index ranged from 0.43 (Chitosan) to 0.51 (CS + FC).

3.3 Colonisation of necrotic tissues by *U. oudemansii*

Sampled tissues from the *U. oudemansii*-treated blocks in 2009 were further examined for *U. oudemansii* incidence and the extent of tissue colonisation by this BCA. The incidence of *U. oudemansii* was significantly higher ($P < 0.05$) on aborted flowers (66%) compared with aborted fruits or the calyptras (26 and 41% respectively), as shown in Fig. 4.

A breakdown of the extent of *U. oudemansii* colonisation on the different tissue types indicated some interesting patterns (Fig. 4). Samples presenting trace quantities represented 60, 77 and 86% of the aborted flowers, aborted fruits and calyptras with *U. oudemansii* present. Samples with low–moderate colonisation were reduced in aborted fruits and calyptras (respectively 5 and 3% of samples) but more abundant in aborted flowers (22% of samples). Moderate colonisation was only observed in aborted flowers (3 and 2% of samples respectively), and only 0.8% of aborted flowers showed high colonisation. This difference in the ability to colonise the different tissue types was also evidenced by the average percentage of colonised tissue (solid diamond in each bar). In aborted flowers, the average percentage of colonised tissue was 6.3% and was significantly higher ($P < 0.05$) compared with the aborted fruits (1.5%) and calyptras (2.4%).

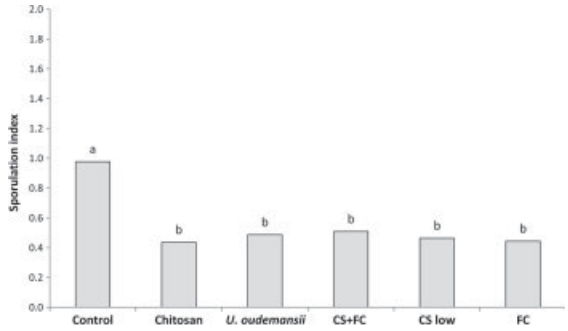

Figure 3. Effect of early-season biologically based treatments on *B. cinerea* sporulation severity in necrotic trash sampled from grape bunches of Macabeu in 2009 in the OLV organic vineyard. Control: untreated; Chitosan: Chitosan 1.44 g L^{-1} + wetting agent 0.5 mL L^{-1} + 1.05 g L^{-1} of $NaHCO_3$; *U. oudemansii*: *U. oudemansii* 2.5 × 10^6 CFU mL^{-1} + wetting agent 0.5 mL L^{-1}; CS + FC: *C. sake* 5 × 10^7 CFU mL^{-1} + Fungicover 50 g L^{-1}; CS low + FC: *C. sake* 1 × 10^7 CFU mL^{-1}; FC: Fungicover 50 g L^{-1}. Represented values are the means of four replicate plots, except for the U. oudemansii and CS + FC treatments which are the means of eight replicate plots. Sporulation was measured visually using a sporulation index (0–5), where 0 = no visible conidiophores on the tissue, 1 = 1–5, 2 = 6–10, 3 = 11–20, 4 = 21–40 and 5 = 40–100. Mean values with the same letter are not significantly different ($P = 0.05$) according to LSD Student's t-test

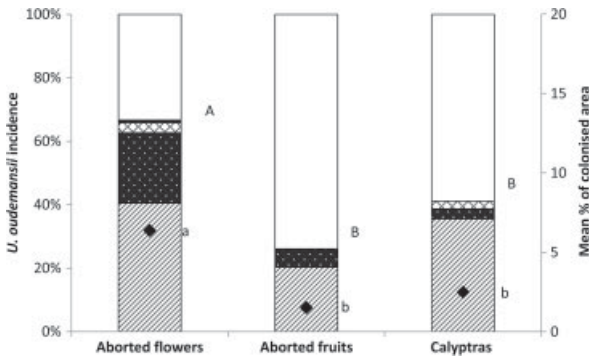

Figure 4. Incidence (bars) and percentage of necrotic tissue colonised by *Ulocladium oudemansii*-like conidiophores (solid diamond shape) from tissue samples removed from grape bunches cv. Macabeu at veraison in 2009. Treated plots received three applications of *U. oudemansii* between 5% flowering and veraison, applied at 2.5 × 10^6 CFU mL^{-1} + 0.5 mL L^{-1} of Mojante Inagra wetting agent. No *U. oudemansii* visible (□) = 0%; trace quantities visible (▨) = 1–5%; low–moderate colonisation (▩) = 6–25%; moderate colonisation (▩) = 26–50%; moderate–high colonisation (■) = 51–75%; high colonisation (■) = 76–100%. Values are means of eight replicates. Mean values with the same upper-case or lower-case letter are not significantly different ($P = 0.05$) according to Tukey's test.

3.4 Reduction of *B. cinerea* incidence in necrotic tissues by early-season biologically based treatments – 2010 field studies

In the 2010 experiment, the average incidence of *B. cinerea* across all tissue types in the untreated control plots was 14% (Fig. 5). The CS + FC and FC treatments significantly reduced *B. cinerea* incidence in necrotic tissues ($P < 0.05$) by 57 and 65% respectively. There were no significant differences between the CS + FC and FC treatments.

3.5 Meterological data, *B. cinerea* in early season and bunch rot at harvest

The temperature, relative humidity, rainfall and leaf wetness data between the early and late part of the growing season and the *B. cinerea* incidence in bunch trash at veraison and BBR at harvest in

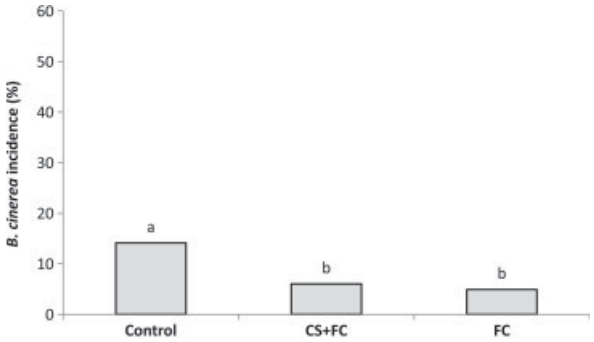

Figure 5. Effect of *Candida sake* and Fungicover on *B. cinerea* incidence on necrotic tissues sampled at 2010 veraison in the OLV organic vineyard. Treatments were applied at 50% flowering and pre-bunch closure. Control: untreated; CS + FC: *C. sake* 5 × 10^7 CFU mL^{-1} + Fungicover 50 g L^{-1}; FC: Fungicover 50 g L^{-1}. Mean values with the same letter are not significantly different ($P = 0.05$) according to LSD Student's t-test.

2009 and 2010 are summarised in Table 2. Rainfall in the early part of the growing season (flowering to veraison) was very similar in both seasons, but there were eight rain episodes in 2009 (Fig. 6) compared with four in 2010. Longer leaf wetness duration most likely accounted for the significantly higher incidence of *B. cinerea* on necrotic floral tissues in 2009 (30–37%) compared with 14% in 2010. However, the high *B. cinerea* inoculum detected in the trash at veraison did not correspond to high BBR severity at harvest, and the significantly lower leaf wetness duration in the late part of the growing season in 2009 (840 min) may have accounted for the lack of BBR development late season.

In contrast, in 2010 there was a low incidence of *B. cinerea* on necrotic bunch trash sampled at veraison, but a substantially longer leaf wetness duration in the late part of the growing season, which resulted in significantly greater BBR severity at harvest in 2010 compared with 2009. Leaf wetness was especially regular in the last 15 days before harvest of the 2010 season, with 13 periods in 2 weeks. Late season in 2009 was hotter and dryer, especially during the first 2 weeks after veraison, and no leaf wetness periods were detected until 14 days before harvest. These findings indicate that late-season wetness duration could be a key driver for BBR development late season compared with *B. cinerea* inoculum potential in the bunch.

4 DISCUSSION

This research represents the first investigation specifically aimed at quantifying the incidence of *B. cinerea* in a range of necrotic tissues trapped within developing grape bunches in a conventional and in an organic vineyard over two growing seasons. It is also the first report on the effect of biologically based treatments on *B. cinerea* saprophytic inoculum sources.

Results showed that a significantly higher overall incidence of *B. cinerea* infection of the bunch trash corresponded to more conducive conditions in the early part of the growing season in 2009 compared with 2010. This was probably related to abundant periods of leaf wetness, as mean temperature, RH and rainfall were similar in both years, while leaf wetness and *B. cinerea* incidence on necrotic tissues were higher in 2009. Under the inland Mediterranean conditions in which the study was carried out, RH is relatively low during the early season, and most of the periods of leaf wetness were directly associated with rain episodes. This suggests that the number of rain episodes, rather than accumulative

Table 2. Incidence of *B. cinerea* on bunch trash, *Botrytis* bunch rot incidence and severity at harvest and key meteorological data of the growing season in 2009 and 2010. Incidence and severity values are from untreated plots

Year	*Botrytis* incidence on bunch trash (%)[a]		*Botrytis* bunch rot incidence (%)	*Botrytis* bunch rot severity (%)	Early season (beginning of flowering to veraison)				Late season (veraison to harvest)			
					RH[b] (%)	*T* (°C)	Rainfall (mm)	Leaf wetness (min)	RH (%)	*T* (°C)	Rainfall (mm)	Leaf wetness (min)
2009	OLV CTD	37.1 a 30.2 a	80.0 a	8.2 b	61.8	22.8	25.0	4020	63.4	22.7	3.0	840
2010	OLV	14.1 b	89.5 a	21.7 a	61.9	22.1	21.7	1324	69.9	21.6	8.5	5252

[a] Values of the same variable linked by the same letter are not significantly different ($P = 0.05$) according to Tukey's test.
[b] RH: relative humidity; *T*: temperature; values are the means of daily mean *T* or RH.

rainfall, could be an important factor in BBR disease development. Few hours are needed by *B. cinerea* to infect grape flowers with the required temperature and RH,[29] and thus isolated rain events could provide favourable conditions for a sufficient time to infect green tissues or necrotic tissues. Moreover, wetness periods inside the bunch last longer, especially in a compact cluster cultivar such as Macabeu,[30] also providing long periods of wetness for saprophytic colonisation. Meteorological conditions also influenced disease development on berries resulting from necrotic tissues from veraison to harvest. In 2009, a high inoculum level at veraison did not correspond to higher bunch rot incidence and severity, while meteorological conditions late in the season favoured disease epidemics in 2010 in spite of a lower inoculum level. An unclear correlation between inoculum level in the bunch and grey mould at harvest was also observed in a 4 year study evaluating 56 field sites in France,[31] suggesting that climate is the more important variation factor. Wolf *et al*. also found a variable response of bunch rot to floral debris removal,[32] stating that other factors were interacting. Further studies are needed for a more precise understanding of the effect of bunch microclimate and meteorological variables on inoculum development inside the grape bunch.

In the present study, aborted fruits presented significantly more *B. cinerea* incidence, representing a significant source of potential inoculum. In other studies, infected aborted fruits were an important source of *B. cinerea* inoculum in the Hunter Valley in Australia,[33] and profuse sporulation on aborted fruits was also reported by Seyb,[6] who identified a significant relationship between the presence of aborted berries within bunches and berry infection at harvest, but not for other trash types, in the dry winegrowing region of Marlborough, New Zealand. However, the importance of aborted fruits for final BBR at harvest is likely to be reduced, as the frequency of aborted fruits in the bunch is generally lower than that of aborted flowers or calyptras. In the present experiments, although there was no exhaustive quantification of tissue types, the 12 aborted fruits per treatment × replicate were difficult to obtain from developing bunches, whereas aborted flowers and calyptras were very abundant. In accordance with these results, calyptras have been identified as the most abundant necrotic tissue inside the bunches in Australian vineyards, and,[34] in addition, the relevance of aborted fruits is also dependent upon the studied cultivar, vine management and several biotic and abiotic factors.[35,36]

In the 2009 studies, three treatment applications before veraison were effective at reducing *B. cinerea* inoculum potential in the calyptras and aborted flowers. In contrast, the reduction in *B. cinerea* on aborted fruitlets was much more variable, and none of the biologically based treatments significantly reduced *B. cinerea* on this inoculum source at veraison in this variety. Nevertheless, as the importance of aborted fruits for BBR is variable, as discussed above, the results suggest that biologically based treatments provide consistent inoculum control early in the growing season.

All treatments were also effective in reducing the *B. cinerea* sporulation index by approximately 50% compared with the untreated control. When samples presenting *B. cinerea* incidence were analysed separately (data not shown), no significant differences in sporulation index were detected, suggesting that sporulation control may be a consequence of incidence reduction rather than a specific effect of treatments on the sporulation process of the pathogen on infected tissues.

The evidence presented in this study further confirms that *U. oudemansii* is an effective antagonist of *B. cinerea* even when applied in comparatively hot and dry Mediterranean vineyard conditions. The findings are similar to those reported on the wine grape variety Sauvignon blanc in cool–temperate viticulture conditions in New Zealand vineyards, where incidence reductions were similar to those of the fungicide treatment.[18] In the present study, *B. cinerea* incidence reduction by *U. oudemansii* applications at the same three key points in the early season was lower (41% compared with 71%), but the incidence of *B. cinerea* in the untreated blocks was higher (37.1% compared with 28.7%). The cited study did not evaluate efficacy for each tissue separately. The ability of *U. oudemansii* to suppress *B. cinerea* is also supported by the significant incidence reduction achieved in calyptras and aborted flowers, although colonisation was categorised as trace or low–moderate for most of the samples with *U. oudemansii*.

The chitosan treatment was only effective in controlling *B. cinerea* incidence in calyptras. Reported modes of action of chitosan against *B. cinerea* range from host defence activation on green tissues[37] to direct antifungal activity of the product solution or the biofilm formed on grape berries.[23,38] After the flower parts senesce and aborted flowers become necrotic, direct antifungal activity is the only likely primary mode of action against *B. cinerea*.

No significant reduction was achieved by CS + FC compared with FC alone in 2009 or 2010 samples, indicating low efficacy of *C. sake* on this substrate. This result reveals interesting information on the mode of action of the *C. sake* and Fungicover combination for reducing grey mould at harvest. *C. sake* is effective at protecting green tissues when combined with FC, while FC by itself has some efficacy against *B. cinerea* infections as well.[24] In addition, FC is able significantly to reduce inoculum sources in the bunch. Possible modes of action of Fungicover include establishment of

a physical barrier,[39] which may prevent infection of senescent and necrotic floral tissues. Fungicover efficacy by itself and the protective effect observed on the yeast antagonist have identified this product as the preferred additive for *C. sake* application.

Although biologically based treatments effectively reduced *B. cinerea* inoculum, the contribution of *B. cinerea* control in necrotic tissues by treatments to the overall reduction in BBR at harvest is difficult to quantify, as it is dependent on meteorological conditions late in the season. Calvo-Garrido *et al*. evaluated the efficacy of the early-season treatments described in this work combined with late-season applications of chitosan or CS + FC.[24] All treatments in that study significantly reduced bunch rot incidence and severity in 2009 and 2010. Nonetheless, only the CS + FC and FC treatments, which consisted of two applications before veraison in 2010, can be examined to evaluate efficacy against bunch rot of the early-season treatments described in this study. Both reduced overall severity, by 85% (CS + FC) and 78% (FC), compared with the control.[24] The reduction rates were very high under favourable conditions for BBR development, confirming the efficacy at harvest of these early-season treatments, although reductions are the consequence of the control on necrotic tissues and other infection pathways not evaluated here, such as latent infections. In spite of the elevated efficacy of these treatments, the control achieved by alternative strategies may be affected by fluctuations in meteorological conditions. Therefore, spray timing may be modified depending on weather conditions during the season. For example, early in the season, preventive applications are desirable prior to rain episodes, whereas applications may be reduced to one at flowering if dry conditions are forecast before veraison. In contrast, inoculum reduction early in the season should be complemented with applications of other post-veraison strategies if meteorological conditions are conducive to infection.

Overall, the present results provide information for a better understanding of *B. cinerea* epidemics with the given cultivar and climatic characteristics, which can be extrapolated to similar warm and dry regions. The number of rain episodes was identified as an interesting indicator of *B. cinerea* development in necrotic tissues before veraison. In addition, aborted fruits showed higher *B. cinerea* incidence and represented the inoculum source most difficult to control by the tested treatments, although the influence of this source for BBR at harvest is variable owing to its low frequency in bunches. Alternative treatments to synthetic fungicides effectively controlled mycelial growth and sporulation of *B. cinerea* on bunch trash under different meteorological conditions. Thus, even if the contribution of early-season treatments to BBR reduction at harvest is dependent on meteorological conditions, the study highlights the potential of these BCAs and NPs as alternative strategies to control BBR of grapes.

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