Environmental effects on the production of *Botrytis cinerea* conidia on different media, grape bunch trash, and mature berries

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Abstract

Background and Aims: The presence of viable sources and of conditions favourable for conidial production, dispersal and deposition is essential for a high number of *Botrytis cinerea* conidia to be available at host infection sites. This study investigated the effect of environmental conditions, growing media and grape organs on the sporulation of ten strains of different genotypes and geographical origin.

Method and Results: Media of different water activity and similar to berry juice at several maturity stages, grape bunch trash or mature berries were inoculated with *B. cinerea* and incubated under variable conditions of temperature and relative humidity. Optimal conditions for sporulation were: temperature between 15 and 20°C, relative humidity >65.5%; water activity=0.971; and medium similar to the juice of softening berries. Conidia produced on bunch trash and mature berries were significantly correlated with those on artificial media.

Conclusions: Differences among strains did not change the response pattern of sporulation to the environmental conditions. Equations were then developed for describing the effect of temperature and relative humidity on sporulation, and of degree-days on the length of latent period.

Significance of the Study: When combined with predictive models for infection, our equations could contribute to the development of an effective disease prediction system for Botrytis bunch rot.

Keywords: epidemiology, grey mould, modelling, Vitis vinifera

Introduction

Botrytis cinerea Pers. is an important pathogen of grapes because of its dual methods of growth, as a necrotrophic invader (van Kan 2006) and a saprophytic coloniser of dead tissue (Jarvis 1977). The fungal population of *B. cinerea* is maintained as a saprophyte throughout the year in the vineyard as well as outside. The saprophytic phase also allows *B. cinerea* to survive when living grape tissues are not available. The fungus produces a large number of conidia on senescing tissues, which can infect susceptible grapevine tissues when present. Following infection, *B. cinerea* becomes a necrotrophic pathogen (van Kan 2006).

There are various factors that are essential for a high inoculum level at the infection sites: (i) viable, productive inoculum sources; and (ii) conditions favourable (a) for conidial production, (b) for their dispersal, and (c) for their deposition on plant surfaces (Holz et al. 2007).

Most *B. cinerea* conidia are probably generated from primary sources of inoculum located within the crop (Elmer and Michailides 2007). The relative importance of different tissues as reservoirs for primary inoculum production in vineyards was recently investigated and reviewed by Mundy et al. (2012a). Rachises, mummified berries and pruned canes have all been identified as potential sources of *B. cinerea* inoculum for the following season (Nair et al. 1995). Rachises are an important inoculum source regardless of their placement in the canopy or on the ground (Seyb 2004). Within the vineyard, the tissue-specific *Botrytis* sporulation ability per rachis has been quantified as 3.9×10^5 to 2.7×10^3 conidia (Jaspers et al. 2013). As

the season progressed, sporulation potential on rachises decreased by at least 40% between capfall and pre-bunch closure (Jaspers et al. 2013), probably as a consequence of rachis degradation over time. When assessed under ideal laboratory conditions, however, spore production on tendrils was low at the start of the season and was reduced further following application of standard fungicide programs used in New Zealand (Mundy et al. 2012b). Carry over inoculum from the previous season explained 70% of variation in flower infection and accounted for only 26% of berry infection (Nair et al. 1995). Bunch trash and leaf trash were the components most likely to provide inoculum for bunch rot at veraison and beyond (Balasubramaniam et al. 1997, Seyb 2004, Calvo-Garrido et al. 2014).

In in vitro experiments, optimal temperature for sporulation was 15°C and production of conidia decreased sharply at higher or lower temperature (Jarvis 1977). Sporulation was observed also at 5 and 10°C (Brooks and Coley 1917). At 24°C, sporulation started about 3 days after inoculation on potato sucrose medium (Shiraishi et al. 1970). *Botrytis cinerea* sporulated better in an unsaturated than in a saturated atmosphere (Paul 1929). In the former atmosphere conidiophores were short and produced numerous conidia while, in the latter, conidiophores were longer (indeterminate growth) and produced fewer conidia (Paul 1929). Conidia generated at the sources of inoculum followed a well-defined diurnal cycle of initiation, production and dissemination that is regulated by fluctuations in temperature and relative humidity (Jarvis 1962). Conidia formation is stimulated by specific wavelengths

of light (Epton and Richmond 1980) and near UV can be used to induce sporulation in culture, although some isolates can sporulate in darkness (Stewart and Long 1987).

A rapid decline in humidity with a rise in temperature in the early morning causes twisting and drying of conidiophores to release conidia into air currents (Jarvis 1962). The concentration of airborne *B. cinerea* conidia in the vineyard showed fluctuations, with daily spore counts ranging from no conidia to peaks of hundreds of conidia per m^3. These fluctuations were related to weather conditions, mainly air temperature and relative humidity (RH) (Aira et al. 2009, Oliveira et al. 2009, Rodriguez-Rajo et al. 2010). Daily average RH close to 90% and a mean air temperature of 20°C coincided with high daily airborne conidia counts. By contrast, the lowest concentration corresponded to a temperature higher than 35°C (Rodriguez-Rajo et al. 2010). Peaks of airborne conidia were also related to rainfall in the previous days (Vercesi and Bisiach, 1982; Oliveira et al. 2009, Rodriguez-Rajo et al. 2010).

Conidia of *B. cinerea* are deposited on berry surfaces primarily from airborne inoculum or in water droplets (Jarvis 1962). Airborne conidia are deposited as single cells (Coertze and Holz 1999) or in small clumps (Harrison and Lowe 1987). Few of the *B. cinerea* conidia dispersed by raindrops become wet enough to enter the droplets, and the majority are carried on the droplet surface as a dry coating (Jarvis 1962). Raindrops may therefore deposit conidia carried on their surfaces as single cells onto berry surfaces during runoff.

Compared to the extensive studies carried out on the sporulation potential of *B. cinerea* on inoculum sources and on diurnal and seasonal patterns of airborne conidia in vineyards, little is known about the effect of environmental conditions, grape organs and medium characteristics on conidia production. This is an important gap in our knowledge, because these factors influence the availability of inoculum during key grapevine growth stages for *B. cinerea* infection (Bulit et al. 1970, Nair and Allen 1993, Broome et al. 1995, Elmer and Michailides 2007).

The aim of this work was to investigate the effect of environmental conditions (i.e. temperature, RH and water activity), medium characteristics (i.e. agar similar to berry juice) and grape organs (i.e. bunch trash and mature berries) on the sporulation of *B. cinerea*. Differently from previous studies, the present work was carried out by using a set of ten *B. cinerea* strains of different geographical origin and belonging to different transposon genotypes (Martinez et al. 2005).

Materials and methods

Fungal isolates and general methods

Ten strains of *B. cinerea* were used, having different geographic origin and issued from the culture collections of the University of Bari, Italy, and from INRA (UMR-Save), Villenave d'Ornon, France (Table 1). All strains were maintained on Potato Dextrose Agar (PDA) (39 g/L) (HiMedia Laboratories, Mumbai, India) at 5°C before being used for the experiments.

For mycelial inoculations of either agar or berries (experiment 1), the strains were cultured on PDA at 20°C in the dark. Plugs of mycelium (4 mm in diameter) were then cut from the edge of 4-day-old colonies with a sterile cork borer, and placed individually in the center of new Petri plates (8.6 cm in diameter) or on the berry equatorial surface.

For conidial inoculation of either bunch trash or berries (experiment 2), Petri plates containing V8-medium [70 g double concentrated tomato (75%), Victoria, SAC, Camagnola, Italy; 100 g soup of legumes, Blédina, Danone, Paris, France; 3 g potassium phosphate dibasic, Difco Laboratories, Detroit, MI, USA; 20 g agar, Difco Laboratories; 1 L distilled water] were inoculated with mycelial plugs (4 mm in diameter) obtained as previously described, incubated at 20°C, and exposed to a 12 h photoperiod using white and near-UV (UV-A at 370 nm) light (Black Light UV-A, L18 w/73, OSRAM, Munich, Germany) for 15–19 days. Conidia were suspended in water by adding into culture plates 7 mL of sterile deionised water containing 0.05% of Tween 20 (polyethylene glycol sorbitan monolaurate, Sigma-Aldrich, St Louis, MO, USA) and gently rubbing the agar surface with a glass rod. The resulting suspensions were filtered through a double layer of sterile cheesecloth to remove remaining mycelial parts. The number of conidia in the suspension was determined and adjusted to 10^4 conidia/mL with a haemocytometer (Bürker, HBG, Giessen, Germany).

Petri plates, bunch trash or berries were incubated in incubators at variable, constant temperature (T) and at varying RH. The various RH values were created in plastic boxes (19×10×5 cm) by placing 100 mL of different saline solutions or distilled water on the bottom of the box (Table 2). Salts were dissolved in boiling distilled water until saturation; solutions were then cooled and salt was added until it stopped dissolving (Winston and Bates, 1960; Dhingra and Sinclair, 1985). Solutions were allowed to stand for 1 week before being used in order to ensure saturation (Winston and Bates, 1960; Dhingra and Sinclair, 1985). The actual RH inside the boxes was checked with a data logger (Tinytag Plus 2, Gemini Data Loggers, Chichester, England) for the entire duration of the experiments.

Table 1. Origin and transposon genotype of the single-spore isolates of *Botrytis cinerea* used in the experiments.

Strain	Plant and organ	Growth stage	Year	Cultivar, location (country)
18.13T	Strawberry fruit	Ripening	2009	Camarosa, Metaponto (IT)
213T	Grapevine leaf	Veraison	1998	Sèmillon, Pessac-Lèognan (FR)
53T	Grapevine berry	Ripening	1998	Sémillon, Sauternes (FR)
344T	Grapevine berry	Ripening	1998	Merlot, Médoc, St-Julien (FR)
18.21V	Strawberry fruit	Ripening	2009	Camarosa, Metaponto (IT)
321V	Grapevine blossom	Bloom	1998	Merlot, Médoc, St-Julien (FR)
351V	Grapevine leaf	Ripening	1998	Merlot, Médoc, St-Julien (FR)
155V	Grapevine leaf	Ripening	1998	Sauvignon, Pessac-Lèognan (FR)
18.1B	Strawberry fruit	Ripening	2009	Camarosa, Metaponto (IT)
18.42F	Strawberry fruit	Ripening	2009	Camarosa, Metaponto (IT)

B, only *Boty* transposon; F, only *Flipper* transposon; IT, Italy; FR, France; T, transposon genotype *transposa*; V, transposon genotype *vacuma*.

Table 2. Effect of temperature on the relative humidity values (±2%) obtained using distilled water or salts to prepare saturated saline solutions†

Salt‡/ distilled water	Relative humidity (%)			
	15°C	20°C	25°C	30°C
NH_4NO_3	70	-	-	-
$NaNO_2$	-	65.5	64	63
NaCl§	-	76	-	-
$(NH_4)_2SO_4$	81	80.5	80	80
$Na_2C_4H_4O_6$	94	92	92	92
Distilled water	100	100	100	100

†Winston and Bates (1960); Dhingra and Sinclair (1985). ‡Carlo Erba Reagents, Milano, Italy. §Only for production of conidia in vitro.

Production of conidia on artificial media

The effect of temperature (5, 10, 15, 20, 25, 30, 35 and 40°C, at 100% RH) and RH (65.5, 76, 80.5, 92, and 100%, at 20°C) on production of conidia of the ten *B. cinerea* strains was assessed in Petri plates containing PDA inoculated with mycelium plugs (as described above). The effect of water activity (0.991, 0.971, 0.955, 0.928, 0.910 a_w at 20°C) on conidia production by the ten *B. cinerea* strains was assessed in Petri plates containing PDA adjusted by adding glycerol (46, 147, 230, 368 and 460 g/L, respectively; Carlo Erba Reagents, Milano, Italy). The plates were inoculated with mycelial plugs as described above. The a_w in the adjusted media was confirmed with the AquaLab CX2T (Decagon Devices, Pullman, WA, USA).

The effect of berry juice composition was assessed in Petri plates containing different media that mimicked the chemical composition of grape berries at the following growth stages (Fregoni 1998): (i) pea-sized berries [stage 75 of Lorenz et al. (1995)]; (ii) veraison (stage 83); (iii) softening of berries (stage 85); (iv) ripe berries (stage 89); and (v) over-ripe berries (stage 93) (Table 3). Media were prepared by adding to the agar (Difco Laboratories) different quantities of sugars (glucose and fructose; Carlo Erba Reagents), organic acids (malic and tartaric acid; Carlo Erba Reagents) and salts (ammonium sulfate, ammonium dihydrogen phosphate, monopotassium phosphate and magnesium sulfate; Carlo Erba Reagents). The pH of the media was adjusted with potassium hydroxide or phosphoric acid (Carlo Erba Reagents) after autoclaving. Agar plates were inoculated with mycelial plugs of four *B. cinerea* strains (18.42F, 18.21V, 18.1B, and 18.13T), sealed with Parafilm, and then incubated at 20°C.

In all the above experiments, five replicate plates were used per treatment (i.e. T, RH, a_w, or media similar to berries). Petri plates were first incubated in darkness at 20°C for 48 h and,

then, incubated at a range of temperature used in experiments and exposed to 12 h of light using both white (TL-D-90, De Luxe 18w/84, Philips, Paris, France) and near UV (Black Light UV-A, 370 nm, L18 w/73, Philips) light. Conidia were collected after 20 days of incubation; 7 mL of sterile water containing 0.05% of Tween 20 were added to Petri plates and the agar surface was gently rubbed with a glass rod. The resulting conidial suspensions were filtered through a double layer of sterile cheesecloth. The density of conidial suspensions was determined with a haemocytometer (Bürker, HBG) and there were two determinations for each suspension. The production of conidia was finally expressed as the number of conidia per cm^2 of colony; the colony area was determined by measuring two perpendicular diameters. All experiments were performed twice.

Production of conidia on bunch trash

Bunch trash was collected at the end of flowering in 2014, in the experimental vineyard (*Vitis vinifera* cv. Barbera) of the University campus, Piacenza, Italy, which was not sprayed with fungicides. Bunch trash was collected by shaking randomly collected bunches, which included calyptras, aborted and damaged fruitlets, and dried complete flowers (Nair et al. 1988).

Trash was dried in an oven at 80°C for 48 h, divided randomly in aliquots of 0.2 g each, and autoclaved at 120°C for 20 min. Each trash aliquot was placed on paper towel in individual Petri plates (6 cm in diameter) and inoculated by uniformly distributing 1 mL of conidial suspension of each of the ten *B. cinerea* strains. After inoculation, Petri plates were incubated: (i) at 15, 20 and 25°C, 100% RH; and (ii) at 100, 92, 80.5, 76 and 65.5% RH, 20°C. There were four Petri plates per fungal strain and temperature or RH combination, and these were incubated for 20 days after which conidia per g of bunch trash were determined as described above. The experiment was performed twice.

Production of conidia on berries

Two experiments (named experiments 1 and 2) were undertaken with ripening berries of *V. vinifera* collected in 2012 in an experimental INRA vineyard (cv. Sauvignon Blanc), (Villenave d'Ornon, France), not sprayed against *B. cinerea*. Details on the characteristics of the vineyard and on the maturity parameters of the berries have been previously published (Ciliberti et al. 2015).

In experiment 1, berries were individually inoculated with mycelium plugs of the four *transposa* and the four *vacuma* strains. Mycelial plugs were placed on the equatorial berry surface, mycelium touching the skin. Berries were then placed on a metallic grid in the plastic boxes, 15 berries per box, and incubated in the dark, at 15, 20, 25, or 30°C, and at four RH levels (Table 2). The experiment was repeated once.

Table 3. Composition of agar-media similar to that of the juice of berries from different growth stages.

Medium	a_w‡	pH	Glucose	Fructose	Malic acid	Tartaric acid (g/L)	Salts§	Agar
Berries pea-sized (stage 75) †	0.991	2	11.25	3.75	31.15	13.85	3.59	20
Veraison (stage 83)	0.987	2.5	33.3	16.6	18.75	6.25	3.59	20
Softening of berries (stage 85)	0.985	3	73	77	11.25	3.75	3.59	20
Ripe berries (stage 89)	0.977	3.5	121.75	128.25	2.5	2.5	3.59	20
Over-ripe berries (stage 93)	0.965	4	155.5	194.5	1	2	3.59	20

†Growth stages according to the scale of Lorenz et al. (1995). ‡The AquaLab CX2T (Decagon Devices, Pullman, WA, USA) was used to check the a_w in the media at 20°C with an accuracy of 0.003. §0.67 g/L $(NH_4)_2SO_4$, 0.67 g/L $NH_4H_2PO_4$, 1.5 g/L KH_2PO_4, 0.75 g/L $MgSO_4 \times 7H_2O$; Carlo Erba Reagents, Milano, Italy.

In experiment 2, berries were placed similarly in plastic boxes containing 100 mL of sterile water on the bottom. Berries were inoculated by putting 10 µL of conidial suspension of the strain 213T on the equatorial area, and boxes were then incubated in the dark, at 20°C for 24 h. Afterwards, 40 µL of calcium hypochlorite (1%, pH 7.2; Carlo Erba Reagents) were put on the inoculated area on the berry surface, in order to stop any further fungal development. After 1 min, the berries were rinsed with 1 mL of sterile water in order to remove the residues of calcium hypochlorite, and kept again in the incubator at 20°C, in the dark, for about 3 weeks to stimulate expression of the Botrytis rot in the inoculated berries. The experiment was repeated once.

In experiment 1, berries were observed at 6, 14 and 22 days post-inoculation (dpi), and in experiment 2 at 6, 9, 12, 14, 16, 19 and 22 dpi. Typical rotten and sporulating berries were detected with the help of a stereoscope (SMZ745 Zoom Stereoscope, Nikon, Tokyo, Japan). Sporulation incidence was then calculated as the proportion of affected berries showing sporulation over the total berries. Length of latency was expressed as the number of days—or degree-days (base 0°C)—elapsed between inoculation and sporulation in individual berries. Degree-days were calculated by multiplying the number of dpi when sporulation first occurred in a berry by the temperature regime at which the berry was incubated.

In experiment 1, the average number of conidia produced was assessed at 22 dpi. Individual sporulated berries were put in distilled water (3 mL) containing 0.05% of Tween 80 and shaken for 30 s. The resulting conidial suspension was managed as described for artificial media to determine the number of conidia per berry.

Data analysis

The proportion of experimental variance accounted for by strains and interactions between strains and the experimental conditions tested (e.g. T, a_w, T×RH, or growing media depending on the experiment) were determined by ANOVA. Numbers of conidia (n) were transformed by using the function ln(n+1) to make the variances homogeneous.

The aim of this work was to study the effect of the influencing experimental factors (i.e. T, RH, a_w, and growing media) on conidial production patterns in different B. cinerea strains rather than to evaluate the differences in sporulation capability of these strains. Therefore, the number of conidia produced by each strain in each treatment were rescaled by dividing these numbers by the maximum (i.e. the value at optimal T, RH, a_w, or growing medium) obtained for that particular strain and condition. These rescaled values (0 to 1) were then independent from the intrinsic capability of each strain to produce conidia.

Quantitative effects of T and RH in the in vitro experiments were analysed by using linear and non-linear regression. Different regression models were used and compared based on the Akaike's Information Criterion (AIC). The model providing the smallest AIC value was then considered the most likely to be correct (Burnham and Anderson, 2002). Regression parameters were then estimated for the average data of rescaled incidence of all strains and for their 95% confidence interval. Deviations of the data for individual strains from the predicted confidence range were then calculated as the absolute difference between the observed value and the predicted either upper or lower range. Finally, the mean absolute error (MAE) and its standard error (s.e.) were calculated. All the statistical analyses were carried out using SPSS (ver. 19, IBM SPSS Statistics, IBM, New York, NY, USA).

Results

### Effect of temperature and relative humidity

Strains varied in their capability to produce conidia, with some strains (18.13T, 18.1B and 18.42F) producing more than 4.8×10^5 conidia per cm^2 of colony at optimal temperature, and other strains (321V, 344T, 155V) producing less than 0.5×10^5 conidia per cm^2 of colony (Figure 1). In the experiment at different temperature values, strain and interaction between strain and temperature accounted for 12 and 18% of the total experimental variance, respectively, while temperature accounted for 66% of variance. In the experiment at different RH levels, strain and interaction accounted for 6 and 2% of total variance, and RH for 87%.

Conidia were produced between 5 and 30°C, and no conidia were produced at either 35 or 40°C. Optimal temperature ranged between 15 and 20°C for all the strains (Figure 1), but distribution patterns of conidial production around the optimum changed with the strain (Figure 1).

The relationship between temperature and conidial production for all strains was fitted by the following bête equation:

$$y = \left[a \times Teq^b \times (1 - Teq)\right]^c, \tag{1}$$

where: y is rescaled conidial production; Teq is an equivalent of temperature calculated as Teq=(T–Tmin)/(Tmax–Tmin), where T is the temperature regime (in °C) and Tmin=0°C and Tmax=35°C are the minimal and maximal temperature for
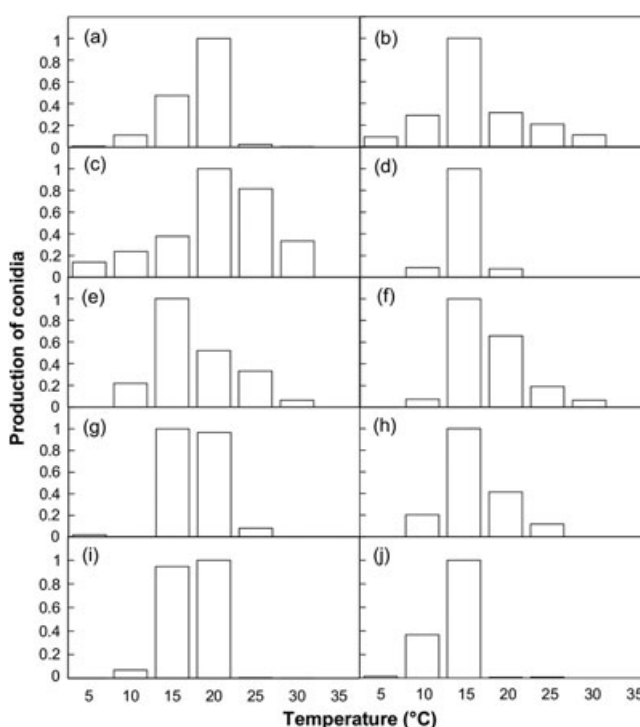
Figure 1. Effect of temperature on rescaled production of conidia by ten *Botrytis cinerea* strains (a) 18.13T, (b) 18.21V, (c) 213T, (d) 321V, (e) 344T, (f) 351V, (g) 53T, (h) 155V, (i) 18.1B and (j) 18.42F (see Table 1 for strain characteristics). Rescaled values were calculated by dividing the number of conidia produced at each temperature on potato dextrose agar by the highest number of conidia found at optimal temperature. The average number of conidia produced per cm^2 of fungal colony at optimal temperature and standard error (of five replicates) are (a) 5.19±1.25, (b) 0.74±0.15, (c) 0.95 ±0.19, (d) 0.15±0.07, (e) 0.11±0.03, (f) 1.24±0.56, (g) 1.09±0.51, (h) 0.04 ±0.02, (i) 7.10±1.32 and (j) 4.84±0.99.

spores to be produced; *a* to *c* are the equation parameters describing the bell-shaped curve of conidial production at changing temperature. Parameters *a*, *b*, and *c* define top, symmetry, and size of the curve, respectively.

Equation 1 provided a good fit of the data (Figure 2a), with low s.e. of the estimated parameters, $R^2 \geq 0.97$, and MAE=0.091±0.019 (Table 4). When the equation for all strains was fitted to the data of individual strains, most (82%) of the deviations from the predicted confidence band were ≤ 0.1 (Figure 2b).

Conidia were produced at all the RH levels and 20°C by all strains, with the exception of strain 53T that produced no conidia at 65.5 and 76% RH (not shown). Production of conidia increased as RH increased from 65.5 to 92%, but slightly decreased at 100% RH (Figure 3a).

The relationship between RH and conidia production was fitted by the following polynomial equation:

$$y = a + b \times RH^2 + c \times RH^3, \tag{2}$$

where: y is rescaled conidial production; RH is the relative humidity level; and *a* to *c* are the equation parameters. This polynomial provided a good fit of the data (Figure 3a), with low s.e. of the estimated parameters, $R^2 \geq 0.85$, and MAE=0.136±0.016 (Table 4). Most (89%) of the deviations of individual strains from the confidence range predicted for all strains were ≤ 0.2 (Figure 3b).

Conidia were produced on bunch trash at all values of temperature and RH tested: there were $1.2 \pm 0.72 \times 10^7$, $7.2 \pm 0.94 \times 10^6$ and $3.0 \pm 0.97 \times 10^6$ conidia/g of bunch trash produced at 15, 20 and 25°C (100% RH), respectively; and $9.85 \pm 1.42 \times 10^5$, $1.06 \pm 0.63 \times 10^6$, $1.09 \pm 0.76 \times 10^6$, $1.2 \pm 0.72 \times 10^7$ and $3.54 \pm 0.48 \times 10^5$ conidia/g of bunch trash were produced at 100, 92, 80.5, 76 and 65.5% RH (20°C), respectively. Pearson correlation coefficients between the ln-transformed numbers of conidia produced on PDA and bunch trash were r=0.65 (*n*=30, *P*<0.001) and r=0.87 (*n*=40, *P*<0.001) for temperature and RH experiments, respectively.

Effect of water activity and berry juice composition
In the experiment with different a_w levels of the growth PDA medium, strains accounted for 12% of the total variance, a_w accounted for 73%, and interaction strain × a_w for 13%. No sporulation was observed at 0.91 a_w (Figure 4). All strains produced conidia at $a_w \geq 0.955$, while four strains (strains 18.13T, 18.21V, 155V and 321V) did not sporulate at 0.928 a_w. Sporulation increased with increasing a_w with maximum at 0.971 a_w, however, numbers of conidia at 0.991 a_w were lower than at 0.971 a_w (Figure 4).

In the experiment on agar-medium similar to berry juice at different phenological stages, the medium accounted for 46% of the total experimental variance, strain and strain × medium for 23 and 16%, respectively. Higher sporulation was observed on the medium similar to softening berries (stage 85) (Figure 5).

Sporulation incidence and production of conidia in berries
In experiment 1, the incidence of rotted berries bearing conidia was affected by RH and temperature (Figure 6a). At 100 and 90% RH, 83 to 99% of the rotted berries bore conidia, while at 80% RH only 50 to 85% of the berries were sporulating. At either 100 and 80% RH, sporulation incidence was higher at 20°C than at 15 or 25°C, while at 90% RH sporulation incidence was >90% at each temperature tested (Figure 6a). In addition, sporulation was faster at 90% RH, 20°C, with about 70% of berries bearing conidia at 7 dpi (Figure 6a). At 80% RH, no sporulation was observed at 7 dpi, and a few only at 14 dpi (Figure 6a).

Similarly, the number of conidia yielded per sporulating berry was higher at 90 and 100% RH than at 80% RH (Figure 6b). In experiment 1, strain accounted for 18% of the total variance in conidial production, RH and temperature for 43 and 2%, respectively. The three interaction effects with strains (temperature × strain, RH × strain, temperature × RH × strain) accounted for approximately 20% of variance.

Length of latent period in berries
The relationship between sporulation incidence and degree-days accumulated (DD, base 0°C) after berry inoculation with either mycelium (experiment 1) or conidia (experiment 2) was fitted by a Gompertz equation 3 in the form:

$$y = 100 \times \exp[-a \times \exp(-b \times DD/10)], \tag{3}$$

where: y=incidence of rotted berries bearing conidia; DD=degree-days (base 0°C) accumulated after berry inoculation; and *a* = 4.19 ± 1.08 and *b* =0.102 ± 0.014 are the equation parameters. This equation provided a good fit of the data (Figure 7), with R^2=0.90. Based on this equation, 50% of the rotted berries produced conidia when 175±45 DD were accumulated following inoculation (Figure 7).

Discussion
The aim of this work was to study the effect on conidial production patterns in *B. cinerea* of environmental conditions (temperature and RH), medium characteristics and grape organs (PDA adjusted to a range of water activity and berry juice composition, bunch trash, and mature berries).
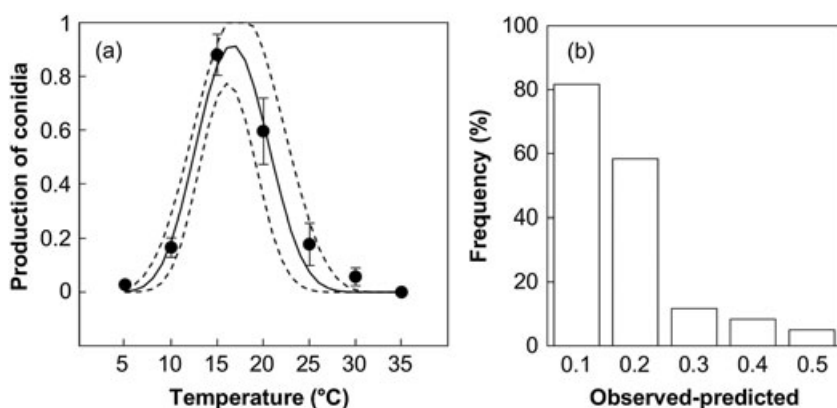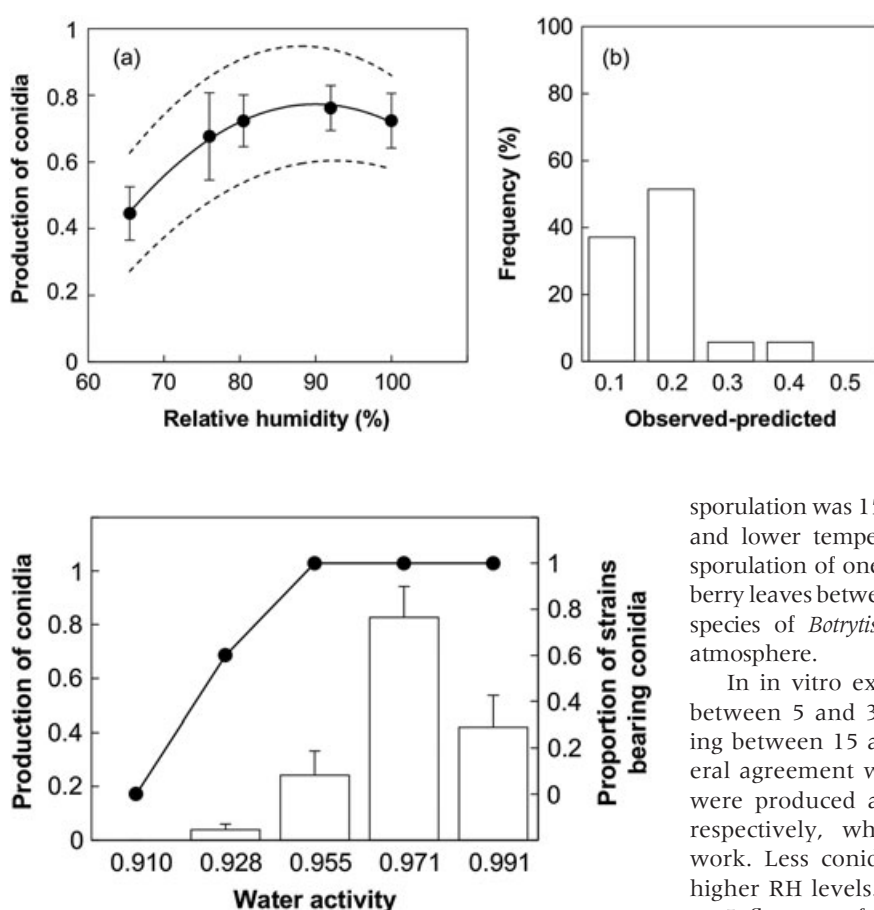

Figure 2. Effect of temperature on rescaled production of conidia by *Botrytis cinerea*. (a) Rescaled values were calculated by dividing the number of conidia produced at each temperature on potato dextrose agar by the highest number of conidia found at optimal temperature. Dots are averages and whiskers the standard errors of ten *B. cinerea* strains. Lines show the fit of data provided by equation 1 for average, minimum and maximum values for the ten strains (see Table 4). (b) Frequency distribution of residuals of the rescaled sporulation predicted by equation 1 (for average data) versus the observed data for the ten strains.

Table 4. Parameter estimates and statistics of the bête and polynomial equations fitting the effect of temperature and relative humidity, respectively, on conidia production in ten strains of *Botrytis cinerea*.

Environmental conditions	Parameter	Average	Lower band	Upper band
Temperature†	*a*	3.70±0.10§	3.54±0.01	3.98±0.15
	b	0.90±0.04	0.85±0.01	0.98±0.05
	c	10.49±1.96	17.38±0.47	6.66±1.33
	R^2	0.97¶	0.99	0.97
	MAE	0.091±0.019	—	—
Relative humidity‡	*a*	-3.539±0.247	-3.896±1.752	-3.291±1.311
	b	0.0957±0.006	0.110±0.043	0.084±0.032
	c	-0.000531±0.0001	-0.001±0.0001	-0.00046±0.00001
	R^2	0.99	0.85	0.92
	MAE	0.136±0.016	—	—

†Model (1): $y = \{[a \times Teq^b \times (1-Teq)]^c\}$; y is sporulation rate; Teq are equivalents of temperature calculated as $(T-Tmin)/(Tmax-Tmin)$, with T=temperature regime, Tmin=0 and Tmax=35°C. ‡Model (2): $y = a + b \times RH^2 + c \times RH^3$; y is sporulation rate; RH is the relative humidity level. §Standard error. ¶R^2 is the coefficient of determination. MAE, Mean absolute error.

Figure 3. Effect of relative humidity (RH) on the rescaled production of conidia by *Botrytis cinerea*. (a) Rescaled values were calculated by dividing the number of conidia produced at each RH level on potato dextrose agar by the highest number of conidia found under the optimal RH condition. The average values (●) and whiskers the standard errors of ten strains. Lines show the fit of data provided by equation 2 for average, minimum and maximum values for the ten *B. cinerea* strains (see Table 4). (b) Frequency distribution of residuals of the rescaled sporulation predicted by equation 2 (for average data) versus the observed data for the ten strains.

Figure 4. Effect of water activity (a_w) on the rescaled production of conidia by *Botrytis cinerea* on potato dextrose agar. Rescaled values were calculated by dividing numbers of conidia produced at each a_w by the highest number of conidia found under the optimal a_w. Bars are averages and whiskers the standard errors of ten *B. cinerea* strains. Line shows the proportion of strains bearing conidia at each a_w.

As far as the authors are aware, there are few studies in the literature concerning the effect of temperature and RH conditions on *B. cinerea* conidia production. Brooks and Coley (1917) considered sporulation of two strains (from apple and peach) between 0 and 30°C. Jarvis (1977) mentioned an unpublished study in which the optimum temperature for sporulation was 15°C, with sporulation falling rapidly at higher and lower temperature. Sosa-Alvarez et al. (1995) studied sporulation of one *B. cinerea* strain from strawberry on strawberry leaves between 10 and 25°C. Paul (1929) found that most species of *Botrytis* sporulated best in a less than saturated atmosphere.

In in vitro experiments, on PDA, sporulation occurred between 5 and 30°C, with the optimal temperature ranging between 15 and 20°C depending on the strain, in general agreement with previous studies. Few and no conidia were produced at a temperature of and exceeding 35°C, respectively, which were not investigated in previous work. Less conidia were produced at 65.5% RH than at higher RH levels.

Influence of a_w has been considered for mycelium growth (Alam et al. 1996; Rousseau and Donéche 2001; Lahlali et al. 2007) but not for sporulation. In this work, sporulation increased with increasing a_w till 0.971 and then decreased at higher a_w (0.991). Optimal a_w for mycelium growth was higher than that for sporulation and, differently from sporulation, no decrease in mycelium growth has been observed at the highest a_w.

In contrast to previous research, ten *B. cinerea* strains belonging to the four transposon genotypes found in vineyards (Martinez et al. 2005; De Miccolis Angelini et al. 2006) were used in this study. Distribution patterns of conidia production over temperature, RH or a_w depended upon the strain. The interaction strain × temperature accounted for a greater part of total variance (18%) than interactions
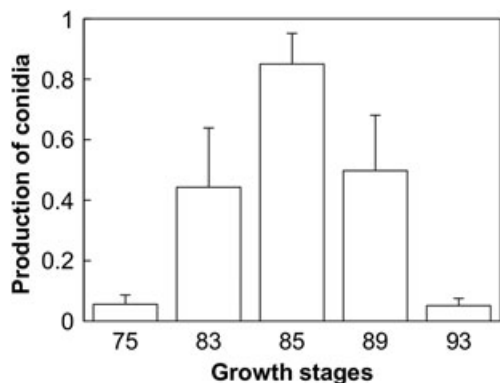
Figure 5. Effect of growth stage on the rescaled production of conidia by *Botrytis cinerea* on agar-media similar to grape berry juice at different growth stages: pea-sized berries [stage 75 of Lorenz et al. (1995)]; veraison (stage 83); softening of berries (stage 85); ripe berries (stage 89); and over-ripe berries (stage 93) (see Table 3 for medium composition). Rescaled values were calculated by dividing the number of conidia produced on each medium by the highest number of conidia found under the optimal medium condition. Bars are averages and whiskers the standard errors of four *B. cinerea* strains.
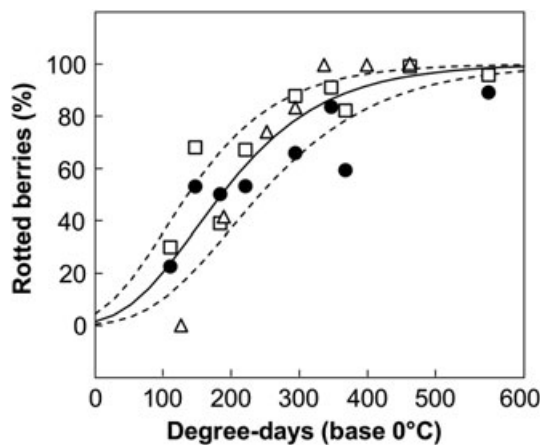
Figure 7. Relationship between incidence of rotted berries bearing conidia in mature Sauvignon Blanc berries and degree-days (base 0°C) accumulated following inoculation with *Botrytis cinerea*. Inoculations were made with mycelium (berries incubated at 15, 20 or 25°C, 100% (●) or 90% (□) relative humidity) or conidia (△) (berries incubated at 20°C, 100% relative humidity) (experiments 1 and 2, respectively). Dotted lines show the 95% confidence band.
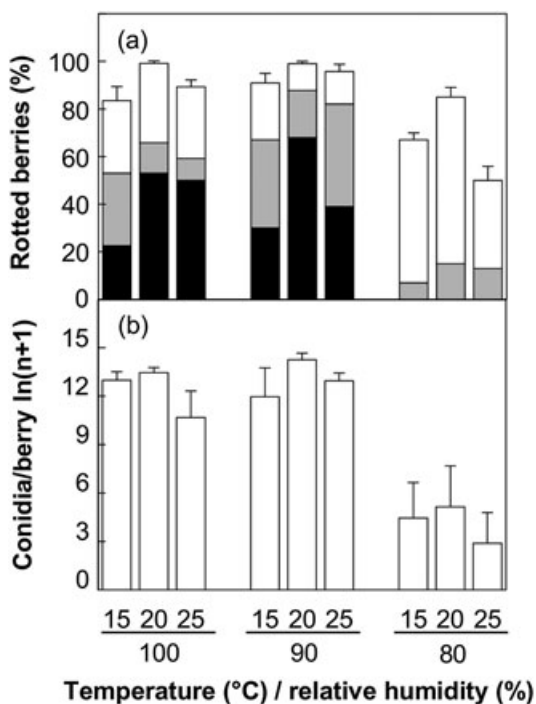
Figure 6. Incidence of rotted berries bearing *Botrytis cinerea* conidia (a) at 7 (■), 14 (▦), and 22 (□) days post-inoculation (dpi) and the number of conidia (b) produced at 22 dpi, in mature Sauvignon Blanc berries inoculated with mycelium of *B. cinerea* at three temperature regimes and three relative humidity levels. In (a) and (b), bars are averages and whiskers are the standard errors for the inoculations carried out with eight *B. cinerea* strains. In (b), numbers are expressed as natural logarithm per sporulating berry.

strain × a_w (13%) and strain × RH (approximately 2%). As these interactions, however, accounted for a minor part of total variance compared to the main factor (i.e. T, RH or a_w), differences among strains did not change the general response pattern of sporulation to temperature, relative humidity and a_w.

In this study, production of conidia on grape bunch trash under a range of temperature and RH conditions was significantly and positively correlated with the production on PDA.

Therefore, data obtained on PDA can be used to infer sporulation patterns of *B. cinerea* on bunch trash. High infection incidence by *B. cinerea*, high sporulation potential, and the close proximity to the developing berries make bunch trash a relevant source of inoculum for berry infection after veraison (Wolf et al. 1997, Seyb 2004). Removal of senescent floral tissues and aborted berries from bunches reduced *B. cinerea* rot by approximately 30% in Merlot grapes (Jermini et al. 1986). In addition to bunch trash, tendrils and leaf pieces were also infected by *B. cinerea* and serve as inoculum sources for berry infection (Nair et al. 1988; Wolf et al. 1997, Seyb 2004). Relative contribution of these inoculum sources to potential sporulation was influenced by the trash type and the grape growth stage. Sporulation potential per mm^2 of tissue was higher for damaged berries and tendrils than for leaf fragments (Jaspers et al. 2013). Sporulation potential incidence (i.e. the number of pieces that sporulated when incubated) for bunch trash ranged from 5 to 40% at pre-bunch closure, and was reduced to less than 5% at veraison (Balasubramaniam et al. 1998). At veraison, 50–100% of leaf trash harboured *B. cinerea* that was able to sporulate after incubation (Balasubramaniam et al. 1997). Factors which may affect the sporulation potential of the trash types include inherent resources or whether the resources have been depleted by previous periods of sporulation (Jaspers et al. 2013). Factors which may affect actual sporulation on these inoculum sources include environmental conditions (Jarvis 1962). Our study contributes to understanding under which environmental conditions such a high sporulation potential may result in high sporulation in the vineyard.

Data on mature berries were also consistent with those found on PDA and bunch trash. Incidence of sporulating berries was higher and conidia were produced at a faster rate at 90% RH than at either 100% or 80% RH (at optimal temperature of 20°C). The number of conidia produced per sporulating berry was higher at 90 and 100% RH than at 80% RH. The existence of an effect of fungal strain on sporulation was also confirmed in these experiments with mature berries, with interactions of temperature, RH and strains accounting for about 20% of total variance. Similar results about temperature effect and quantity of water

available in the air have been reported on grapevine (Thomas et al. 1988), tomato (O'Neill et al. 1997), strawberry (Sosa-Alvarez et al. 1995), and lentil (Davidson and Krysinska-Kaczmarek 2007).

Production of *B. cinerea* conidia was also influenced by the chemical composition of the media similar to berry juice. Higher sporulation was observed on the medium similar to softening berries [stage 85 of the scale of Lorenz et al. (1995)] than for both the previous and following growth stages. These results were almost consistent among the *B. cinerea* strains. To our knowledge, there are no similar published studies. The stimulator or inhibitory effect of several compounds on the growth of *B. cinerea* has been extensively studied (Kosuge and Hewitt 1964, Nyerges et al. 1975, Doneche 1986, Padgett and Morrison 1990). Our results may indicate that, in addition to temperature and humidity, sporulation potential also depends on the growth stage of the infected berries. Further studies are necessary to clarify which causes may account for these differences and to confirm these finding by using berries at different growth stages to verify whether the presence of anti-fungal compounds in the host tissue may inhibit sporulation as they do for germination and infection (Nyerges et al. 1975, Langcake 1981).

Our results provide quantitative data on the effect of temperature and RH conditions on the sporulation of *B. cinerea* on grape bunch trash and mature berries. Two equations were developed to describe the effect of temperature and RH on rescaled production of conidia. For any temperature or RH values, these equations provide a prediction interval which accounts for strain variability in response to the environment. A third equation was developed to determine the length of latent period (i.e. the time elapsed between inoculation and initiation of sporulation) as a function of the degree-days (base 0°C) accumulated after inoculation. The latent period of *B. cinerea* in grape berries has been previously neglected in the literature, even though it is an important component of epidemic development (Jarvis 1980) and fitness (Pringle and Taylor, 2002). In contrast, the latent period was studied on strawberry leaves (Sosa-Alvarez et al. 1995), tomato stems (Dik and Wubben 2007) and *Primula × polyantha* plants (Barnes and Shaw 2002), as for other *Botrytis* species (Bouhassan et al. 2004; Soares et al. 2010, Sehajpal and Singh 2014).

Conclusion

Equations developed in this work may be of value in predicting sporulation events of *B. cinerea* and identifying periods when the presence of conidia in the air may be high. Epidemics of grey mould did not occur if the concentration of airborne conidia were 40 conidia per m^3 of air or less, despite the occurrence of suitable conditions for infection (Bulit et al. 1970, Carisse et al. 2014). Thus the common assumption that *B. cinerea* inoculum is always available to cause infection in the vineyards has been considered too conservative for an effective management of Botrytis bunch rot (Ellison et al. 1998). Most forecasters of plant disease currently in use, including those for *B. cinerea* on grape, are generally simple and based on one component of the disease cycle (Madden and Ellis 1988). Infection is the most successfully and commonly used component of the disease cycle, although relying on it, alone, may be insufficient for accurate predictions. In the absence of inoculum, predicting disease occurrence based on infection periods would be ineffective and may result in excessive control applications if conditions

do not favour other components (Madden and Ellis 1988). Therefore, when combined with predictive models for infection (Ciliberti et al. 2014, 2015) our equations could contribute to the development of an effective disease prediction system.

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