Influence of Fungal Strain, Temperature, and Wetness Duration on Infection of Grapevine Inflorescences and Young Berry Clusters by *Botrytis cinerea*

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ABSTRACT

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The effect of temperature and wetness duration on infection of *Vitis vinifera* inflorescences (from "inflorescence clearly visible" to "end of flowering" stages) and young berry clusters (at "fruit swelling" and "berries groat-sized" stages) by *Botrytis cinerea* was investigated. Artificial inoculations were carried out using conidial suspensions of eight *B. cinerea* strains belonging to the transposon genotypes *transposa* and *vacuma*. Infection incidence was significantly affected by strain but not by transposon genotype (transposon genotype accounted for only 6.5% of the variance). Infection incidence was also affected by the interaction between strain and growth stage of the inflorescence or berry cluster (overall accounting for approximately 57% of the experimental variance). Thus, under our experimental conditions, the ability to cause infection was a strain rather than a transposon genotype attribute. Across all strains, infection incidence was lowest when inflorescences were clearly visible or fully developed, highest at flowering (from beginning to end of flowering), and intermediate at the postflowering fruit stages (fruit swelling and berries groat-sized). One *transposa* strain, however, was highly virulent on all grapevine growth stages tested. The effects of temperature and wetness duration on infection incidence were similar for all fungal strains and grapevine growth stages; infection incidence was highest at 20°C and lowest at 30°C, and was also low at 5°C. Similar results were obtained for mycelial growth and conidial germination. Based on the pooled data for all strains and grapevine growth stages, an equation was developed that accounted for the combined effects of temperature and wetness duration on relative infection incidence. This equation should be useful for developing decision-making systems concerning *B. cinerea* control at early grapevine growth stages.

Additional keywords: transposable elements.

Botrytis cinerea Pers., the anamorph of *Botryotinia fuckeliana* (de Bary) Whetzel, is a widespread fungus that attacks over 200 different plant species, including grapevine (36). Infection of grapevine by *Botrytis cinerea* before harvest can seriously reduce fruit yield and wine quality (42). The fungus is also the causal agent of Botrytis bunch rot, which develops mostly from veraison onward (i.e., during berry ripening). Disease symptoms in the early season (i.e., until end of blooming) include necrotic brown spots on leaves and inflorescences. Before bloom, *B. cinerea* can invade developing inflorescences, and severe infections may cause entire inflorescences to dry and fall off. During the bloom period, flower parts and individual flowers can become infected. *B. cinerea* can infect flower stigmas and stylar tissues (53,60). Moreover, the fungus can infect stamens and then grow basipetally into the receptacle and vascular tissue (62,75). The susceptibility of flowers to infection by *B. cinerea* is high because the low resveratrol content (39) and abundance of pollen (16,45). At the end of bloom or just after bloom, the fungus can develop on flowerhoods (or calyptras), stamens, and aborted berries still attached to the cluster. From there, *B. cinerea* can attack the pedicel or the rachis, forming small brown rotted lesions that eventually turn completely black (32,33).

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In addition to causing inflorescence and blossom blight, infections at bloom may remain latent. After infecting flower parts or developing ovaries, *B. cinerea* may be inhibited by the natural resistance of the green berries (3,6,30,32,37,39,53,63,69). Then, during berry ripening, the pathogen may resume growth and cause bunch rot. Latent infection of fruit occurring from flowering onward is an important pathway for the subsequent development of fruit infection (21,31,65). Other infection pathways are also related to blooming in that *B. cinerea* can survive as a saprobe on floral debris within bunches and, after veraison, its mycelia or conidia may subsequently infect the ripening fruit (21). Floral debris has been considered a major source of inoculum within developing bunches (14,60,67). After veraison, berry susceptibility to the pathogen increases with fruit maturation (19). As berries mature, increasing berry exudates may reactivate saprophytic growth of mycelium on debris, which then triggers infection of berries. At the same time, conidia produced inside the clusters by sporulation on floral debris are in an ideal position to infect berries when host and environmental conditions are favorable (66).

Therefore, even though Botrytis bunch rot appears and develops relatively late in the growing season, early season infection by *B. cinerea* plays a key role in the disease development, as demonstrated by field studies involving artificial inoculation between the time of flowering and the development of pea-sized berries (39,53,62,63,75). Despite this key role, few studies have been published on weather conditions favoring infection of grapevine at early growth stages. Nair and Allen (58) studied how infection of grape flowers is affected by wetness duration (WD) and

temperature. They found that, at the optimum temperature for flower infection (23.7°C), flowers become infected after only a brief exposure to wetness (i.e., 63% of flowers were infected after only 1.3 h of wetness).

In addition to the insufficient knowledge concerning the effect of environmental conditions on floral infection, information is also lacking concerning the role of the fungal transposon genotype on infection at these early growth stages. The species *B. cinerea* sensu stricto (23,76) is composed of four transposon genotypes: (i) *transposa* (containing both Boty and Flipper transposable elements), (ii) *flipper*-only (containing only Flipper), (iii) *boty*-only (containing only Boty), and (iv) *vacuma* (containing neither Boty nor Flipper) (2,17,22,26,34,48,51,57).

Frequencies of strains belonging to the different transposon genotypes in the fungal populations from vineyards were found to be highly dependent on geographic location (57,71), isolation year (71), and sampling time during the season (18,51,52). The frequency of *vacuma* genotypes usually peaks at flowering and then decreases from blossom to harvest. The *transposa* genotypes are frequent during all parts of the growing season and peak after veraison and particularly on mature berries. Martinez et al. (52) suggested that *vacuma* strains are more saprotrophic than *transposa* strains, the latter being more virulent on grape berries at different maturation stages. This hypothesis would account for the high frequency of *vacuma* genotypes at the end of flowering on floral debris and the prevalence of *transposa* genotypes on maturing berries (52). Recent studies showed that *B. cinerea* transposable elements are involved in the production of small RNAs that silence the expression of host defense genes (5,77). However, the ability of the *B. cinerea* strains of the different transposon genotypes to infect inflorescences at different stages, and especially before and at flowering, remains unknown.

In the present study, we investigated the effect of temperature and WD on *B. cinerea* infection of *Vitis vinifera* inflorescences (from inflorescence visible to full flowering) and berry clusters (at fruit swelling and when berries are groat-sized). Different fungal strains belonging to the transposon genotypes *transposa* and *vacuma* were used to determine whether transposon genotype influences the incidence of infection or the relationship between temperature and WD. The effects of temperature on mycelial growth and conidial germination of these fungal strains were also investigated.

MATERIALS AND METHODS

Fungal isolates and culture conditions. Experiments were performed with eight single-spore strains belonging to *vacuma* and *transposa* genotypes within the species *B. cinerea* (Table 1). The strains differed in geographic origin and were obtained from the culture collections of the University of Bari, Italy, and from INRA (UMR-Save) in Villenave d'Ornon, France. Strains were kept on potato dextrose agar (PDA) at 39 g liter^{-1} (HiMedia Laboratories, Mumbai, India) at 5°C before use.

The conidial suspensions used for germination and infection assays were obtained as follows. The strains were cultured in petri dishes (8.6 cm in diameter) containing PDA at 20°C and exposed to 12 h of light using both white (TL-D-90 De Luxe, 18 W; Philips, Paris) and near-UV (TL-D-08 Blacklight Blu, 18 W; Philips) light for 20 days. Afterward, conidia were suspended in water by adding 7 ml of sterile deionized water containing 0.05% Tween 20 (polyethylene glycol sorbitan monolaurate; Sigma-Aldrich, St. Louis) to each culture 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 mycelia. The number of conidia in the suspension was determined with a hemocytometer (Bünker, HBG, Germany) and was adjusted to 10^6 and 10^5 conidia ml^{-1} for conidial germination and infection potential bioassays, respectively.

For colony growth assays, the strains were cultured on PDA at 20°C in the dark. Plugs of mycelium (4 mm in diameter) were cut from the edge of 4-day-old colonies with a cork borer and placed individually in the center of new petri dishes.

Conidial germination. Aliquots of 10 µl of the conidial suspension were uniformly plated on plugs (4 mm in diameter and 5 mm thick) of AG medium (agar at 20 g liter^{-1} [Difco Laboratories, Detroit] and glucose at 10 g liter^{-1} [Carlo Erba Reagents, Milano, Italy]). Three replicated agar plugs were placed on a microscope slide. Microscope slides were then put in petri dishes (8.6 cm in diameter) containing 1 ml of sterile water. The slides were placed on a 5-mm-high pedestal so that they were not in contact with water. The dishes were sealed with Parafilm to maintain a saturated atmosphere and were incubated at 5, 10, 15, 20, 25, 30, 35, or 40°C in darkness. Conidial germination was stopped after 0, 1, 3, 6, 9, 12, and 24 h by application of lactophenol methyl blue (200 mg of phenol, 0.5 mg of cotton blue, 400 ml of glycerol, 200 ml of lactic acid, and 200 ml of distilled water) (Carlo Erba Reagents). One hundred conidia per replicate (i.e., per agar plug) were observed at ×40 magnification with a microscope, with three replicates for each combination of strain, temperature, and time. The proportion of conidia germinated was determined (conidia were considered germinated when germ tubes were visible). The experiment was performed twice.

Mycelial growth. Petri dishes (8.6 cm in diameter) containing PDA were inoculated with mycelium plugs as previously described and incubated at 5, 10, 15, 20, 25, 30, or 35°C in darkness for 20 days. Three replicate plates for each combination of strain and temperature were assessed. Two perpendicular diameters of each colony were measured every 2 days and were used to calculate the average diameter of the colony. The relative mycelial growth was then calculated by dividing the average colony diameter by the diameter of the petri dish. The experiment was performed twice.

Inflorescence and cluster infection. For infection assays, inflorescences and young clusters were collected in a 20-year-old vineyard ('Barbera' grape) that had not been sprayed against *B. cinerea* and that was located at Ziano Piacentino, North Italy (latitude 44°59′25″, longitude 9°23′50″). The plants, which were trained to a cordon spurred system, were spaced 1 m within rows and 2 m between rows. Two infection experiments were carried out over 2 years, as described in the following paragraphs.

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

Strain[a]	Plant and organ	Growth stage	Year	Cultivar, location
18.13T	Strawberry fruit	Ripening	2009	Camarosa, Metaponto, Italy
213T	Grapevine leaf	Veraison	1998	Sèmillon, Pessac-Lèognan, France
53T	Grapevine berry	Ripening	1998	Sémillon, Sauternes, France
344T	Grapevine berry	Ripening	1998	Merlot noir, Médoc, St-Julien, France
18,21V	Strawberry fruit	Ripening	2009	Camarosa, Metaponto, Italy
321V	Grapevine blossom	Bloom	1998	Merlot noir, Médoc, St-Julien, France
351V	Grapevine leaf	Ripening	1998	Merlot noir, Médoc, St-Julien, France
155V	Grapevine leaf	Ripening	1998	Sauvignon, Pessac-Léognan, France

[a] T = *transposa* transposon genotype and V = *vacuma* transposon genotype.

In the first experiment, inflorescences and young berry clusters were collected at seven growth stages, which were numbered according to the scale of Lorenz et al. (47): (i) inflorescence clearly visible (stage 53); (ii) inflorescence fully developed (stage 57); (iii) beginning of flowering, 10% of flowerhoods fallen (stage 61); (iv) full flowering, 50% of flowerhoods fallen (stage 65); (v) end of flowering (stage 69); (vi) fruit set, young fruit beginning to swell (stage 71); and (vii) berries groat-sized (stage 73). In the laboratory, the inflorescences or clusters were divided into pieces of uniform size (approximately 2 cm long). These pieces were rinsed under running tap water for 10 min and then disinfested for 5 min in 70% ethanol (Carlo Erba Reagents), for 3 min in calcium hypochlorite (50 g liter^{-1}, adjusted at pH 7.2 with acetic acid) (Carlo Erba Reagents), and, finally, for 2 min in 70% ethanol. After they were rinsed three times with sterile water and then dried under a laminar flow, inflorescence or cluster pieces were inoculated by immersion in one conidial suspension of each of the eight fungal strains, prepared as described above. The inoculated pieces were then placed in petri dishes (8.6 cm in diameter), five pieces per dish, containing a metallic grid on two filter papers soaked with sterile water. The dishes were sealed with Parafilm to maintain a saturated atmosphere and then placed at 20°C in darkness. After 0, 3, 6, 12, 24, and 48 h, the pieces were disinfested with calcium hypochlorite (10 g liter^{-1}, adjusted at pH 7.2 with acetic acid) for 2 min to remove any viable inoculum from the surface, rinsed by immersion in sterile water two times, and then dried under a laminar flow. Dried pieces were then placed in other petri dishes, which were prepared as described above and which were incubated at 20°C in darkness. These pieces were periodically observed over 2 weeks with a stereomicroscope to detect the presence of typical *B. cinerea* sporulation as evidence that infection had occurred. Infection incidence at 2 weeks postinoculation was then calculated as the proportion of inflorescence or cluster pieces showing *B. cinerea* sporulation. Three replicates (10 pieces per replicate) were considered for each combination of fungal strain, grapevine growth stage, and hours of WD (i.e., the time between inoculation and the disinfection with calcium hypochlorite). The experiment was performed twice.

In the second experiment, inflorescences and berry clusters were collected at three growth stages: (i) inflorescence swelling, flowers closely pressed together (stage 55); (ii) full flowering, 50% of flowerhoods fallen (stage 65); and (iii) berries groat-sized (stage 73). In the laboratory, inflorescence or cluster pieces were processed as previously described until they were inoculated with fungal strain 18.13T or 18.21V. The inoculated inflorescence or cluster pieces were incubated at 5, 10, 15, 20, 25, or 30°C in darkness for 0, 3, 6, 12, 24, and 48 h of WD. Inflorescence or cluster pieces were then processed and assessed for infection incidence as previously described. Three replicates (10 pieces per replicate) were considered for each combination of fungal strain, grapevine growth stage, temperature, and hours of WD. The experiment was performed twice.

In both experiments, additional inflorescence and cluster pieces were treated with sterile water rather than with conidial suspensions and were placed in petri dishes in a saturated atmosphere for 72 h in darkness. This treatment, which corresponded to a control, indicated any natural infection by *B. cinerea* that occurred in the vineyard before sampling.

Data analysis. *Analysis of variance*. The incidence data from the first infection experiment were used to calculate the area under the infection progress curve (AUIPC) (15). In a factorial analysis of variance (ANOVA), AUIPC values were used to test the effect of transposon genotype, strains within genotype, grapevine growth stage at the time of inoculation, and their interactions. In the ANOVA, transposon genotypes and growth stages were tested as fixed effects and strains as random effects. The protected Fisher's least square difference test was used at $P = 0.05$ to discriminate between means. The ANOVA was performed using DSAASTAT (ver. 1.101; Dipartimento di Scienze Agrarie ed Ambientali, Perugia, IT).

Selection of the regression model. Data on conidial germination and relative mycelial growth were regressed against temperature and incubation time. Data from the second experiment on infection incidence on inflorescences and berry clusters were regressed against temperature and WD. Infection incidence data of any strain and growth stage were rescaled by dividing each value by the infection incidence of that particular strain and grapevine growth stage after 48 h at 20°C (i.e., the maximum value obtained). Rescaled values were then independent from the capability of the strain to cause infection and enabled the comparison among strains of the combined effect of temperature and WD.

Different nonlinear regression models were compared based on the Akaike's Information Criterion (AIC). The following model, which provided the smallest AIC values, was then considered the most likely to be correct (13):

$$y = [a \times \mathrm{Teq}^{b} \times (1 - \mathrm{Teq})]^{c}/[1 + \exp(d - e \times x)] \quad (1)$$

where y = the relative mycelial growth, proportion of germinated conidia, or rescaled infection incidence; Teq = equivalents of temperature calculated as (T – Tmin)/(Tmax – Tmin), where T is the temperature regime (°C) and Tmin and Tmax are minimal and maximal temperature for growth, germination, or infection, respectively; x = incubation time in days for mycelial growth, in hours for conidial germination, and in hours of wetness for infection; a to c = the equation parameters accounting for the effect of temperature; and d and e = the equation parameters accounting for the effect of time. Tmin and Tmax were considered as equation parameters and estimated accordingly (81). The equation parameters were estimated using the nonlinear regression procedure of SPSS, which minimizes the residual sums of squares using the Marquardt algorithm (SPSS ver. 21; IBM SPSS Statistics, IBM Corp., New York).

Model 1 includes an S-shaped curve that describes growth, germination, or infection over time; the curve is represented by a logistic equation at the denominator (with the parameter d defining the lag phase and the parameter e being the growth rate of the curve), which has an asymptotic value that depends on temperature according to a bell-shaped, bête equation at the numerator (with parameters a, b, and c, which define the top, symmetry, and size of the curve, respectively).

Measurement of model accuracy. The concordance correlation coefficient (CCC) was calculated as a measure of model accuracy (49). This is the product of two terms: the Pearson product-moment correlation coefficient between observed and predicted values and the coefficient C_b, which is an indication of the difference between the best fitting line and the perfect agreement line (if CCC = 1, the agreement is perfect). The mean absolute error (MAE) was used to measure how close model predictions were to the real data. MAE has the same units as the original data and should be as low as possible (80). The model efficacy (EF) was calculated as the ratio of the mean square error to the variance in the observed data, subtracted from unity (when the error is zero, EF = 1 and the equation provides a perfect fit) (61). The W index of agreement is the ratio between mean square error and total potential error, with W = 1 representing a perfect fit (79). The coefficient of residual mass (CRM) is a measure of the model tendency to overestimate or underestimate the observed values (a negative CRM indicates a tendency toward overestimation) (46).

Comparison of fitted curves. The extra sum-of-squares F test was used to compare the curves fitted with model 1 for the different fungal strains, as indicated by Motulsky and Christopoulos (55). The null hypothesis H0 was that one curve fit all the data

points of two strains and that the difference observed was purely due to chance; the alternative hypothesis was that the curves of two strains were different. Model 1 was fitted to all the data of two strains, and estimates for each of the five parameters in the equation were obtained. The sum-of-squares (SScomb) and the df (dfconb = n values – n parameters) of the combined equation were also calculated. The data sets of the two strains were also fitted separately to obtain two distinct curves with five parameters at each time; the sum-of-squares of the two separate curves were added (SSsep) as well as the df (dfsep). The differences "SScom – Sssep" and "dfcomb – dfsep" were calculated and used to calculate the F ratio against the alternative hypothesis (separate curves); the corresponding P value was calculated.

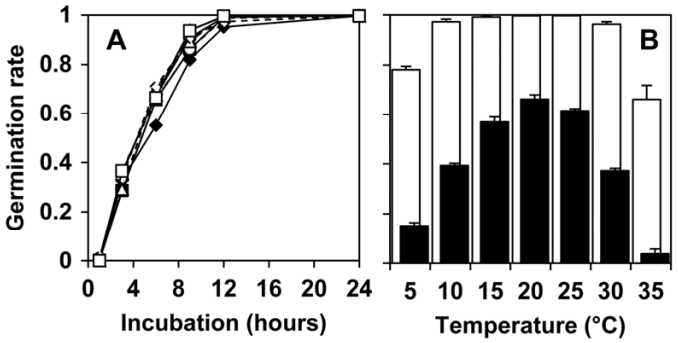
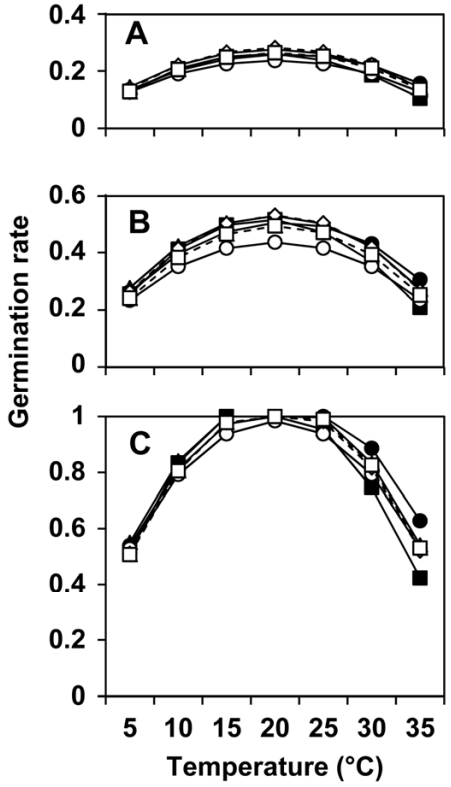


Fig. 1. Conidial germination rate for eight *Botrytis cinerea* strains (■ = 18.13T, ◆ = 53T, ▲ = 213T, ● = 344T, ○ = 18.21V, Δ = 155V, ◇ = 321V, □ = 351V) as affected by time and temperature. **A,** Germination at 20°C over time. **B,** Germination (mean + standard error for all eight strains) at 5 to 35°C after 6 h (black bars) and 24 h (white bars); no germination occurred at 40°C. Conidia were germinated in saturated atmosphere.



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The above procedure was repeated for any combination of fungal strains. An overall equation (for all the strains) was finally calculated because no significant differences existed for any combination of strains.

RESULTS

Influence of temperature on conidial germination in vitro. Regardless of fungal strain, conidia germinated rapidly at 20°C. Approximately one-third had germinated after 3 h, two-thirds after 6 h, and nearly all after 12 h at 20°C (Fig. 1A). At lower or higher temperatures, germination was slower but almost all conidia had germinated after 24 h at temperatures between 10 and 30°C. No germination was observed at 40°C (Fig. 1B).

Model 1 provided a good fit of conidial germination data when 0 and 40°C were used as minimal and maximal temperatures for germination, with R^2 values of 0.94 to 0.95 depending on fungal strain (data not shown). Estimates for the model parameters were between 3.959 and 4.989 for a, 0.884 and 1.118 for b, 0.663 and 1.123 for c, 2.941 and 3.261 for d, and 0.437 and 0.556 for e. The standard errors of these estimates were at least 10 times lower than the parameter value (data not shown). Curves fitted for the single strains did not significantly differ from each other according to the F test, with the greatest F value (F = 0.03, P = 0.99) for the comparison between two strains belonging to the transposon genotype *transposa*, 53T and 344T. Therefore, the effect of temperature on the dynamics of conidial germination was similar for the different strains (Fig. 2).

The overall equation for aggregate strains had the following parameter estimates: a = 4.414 ± 0.097, b = 0.982 ± 0.022, c = 0.852 ± 0.036, d = 3.089 ± 0.1115, and e = 0.490 ± 0.020, with R^2 = 0.94. The comparison of predicted versus observed conidial germination provided a very low error (MAE = 0.06) and CCC = 0.97. This CCC value was close to 1, corresponding to perfect agreement. The other indices of goodness-of-fit were also close to 1 EF = 0.94, W = 0.98). A CRM = –0.02 indicated a slight overestimation.

Influence of temperature on mycelial growth in vitro. At the optimal temperature of 20°C, the fungal colonies reached the maximum diameter within 4 to 6 days, with no relevant differences between strains, except that strain 213T grew more slowly than the other strains (Fig. 3A). Mycelium grew at all the temperature regimes tested, with the optimum at 20°C and the minimum at 35°C (Fig. 3B).

Model 1 provided a good fit of the observed mycelial growth data with Tmin = 0°C and Tmax = 40°C, and with R^2 of 0.85 to 0.90 according to the strain (data not shown). Estimates for the

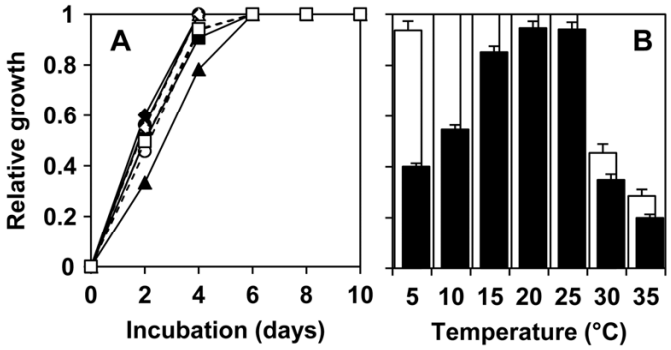

Fig. 3. Relative growth of eight *Botrytis cinerea* strains (■ = 18.13T, ◆ = 53T, ▲ = 213T, ● = 344T, ○ = 18.21V, Δ = 155V, ◇ = 321V, □ = 351V) on potato dextrose agar as affected by time and temperature. **A,** Relative growth at 20°C over time. **B,** Relative growth (mean + standard error for all eight strains) after 4 days (black bars) and 8 days (white bars) at 5 to 35°C. Relative growth was calculated by dividing the diameter of the colony by the diameter of the petri dish in which the colonies were growing.

model parameters were between 3.652 and 4.069 for a, 0.820 and 0.950 for b, 0.306 and 0.618 for c, 2.323 and 2.799 for d, and 0.823 and 1.088 for e. The standard errors of these estimates were at least 10 times lower than the parameter value (data not shown). Based on these equations, mycelial growth in response to temperature followed a similar pattern for all strains but the different strains grew at different speeds (Fig. 4). The growth rate until the colony reached one-half of the maximum growth (i.e., the diameter of the petri dish) ranged from 1.34 mm/day (strain 213T) to 1.89 mm/day (strain 155V) at the optimal temperature of 20°C (Fig. 4).

Curves fitted for the single strains did not significantly differ from each other according to the F test: the greatest F value (0.28) corresponded to the comparison between strains 155V and 213T (P = 0.92). For all strains, the overall equation had the following parameter estimates: a = 3.782 ± 0.192, b = 0.900 ± 0.057, c = 0.475 ± 0.070, d = 2.485 ± 0.225, and e = 0.936 ± 0.082, with R^2 = 0.87. Comparison of predicted versus observed mycelial growth showed a good agreement: CCC = 0.87, MAE = 0.12, EF = 0.7, and W = 0.98. A CRM = –0.01 indicated no substantial overestimation by the model.

Influence of the grapevine growth stage on infection. The AUIPC was significantly influenced by strain within each transposon genotype (P < 0.001), by the grapevine growth stage at the time of inoculation (P < 0.001), and by the interaction between fungal strain within transposon genotype and growth stage (P < 0.001). These sources of variation accounted for 19.9, 31.7, and 36.9% of the total variance, respectively. However, the transposon genotype (*transposa* or *vacuma*) accounted for only 6.5% of the total variance.

The average AUIPC for all strains was higher for inflorescences inoculated during flowering (stages 61, 65, and 69) than for inflorescences inoculated after flowering (stages 71 and 73). AUIPCs were lower when developing inflorescences (stages 53 and 57) were inoculated than when other stages were inoculated

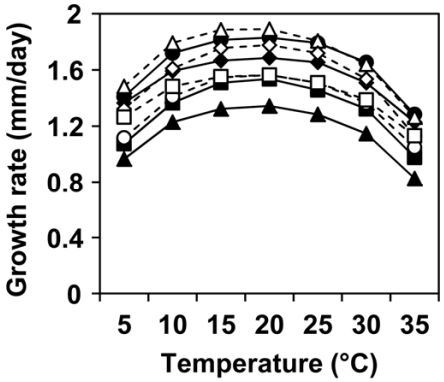

Fig. 4. Growth rate of eight *Botrytis cinerea* strains (■ = 18.13T, ◆ = 53T, ▲ = 213T, ● = 344T, ○ = 18.21V, Δ = 155V, ◇ = 321V, □ = 351V) on potato dextrose agar as affected by temperature. Growth rate is expressed as the average rate (mm/day) required to reach 50% of the maximal colony diameter, as fitted by model 1.

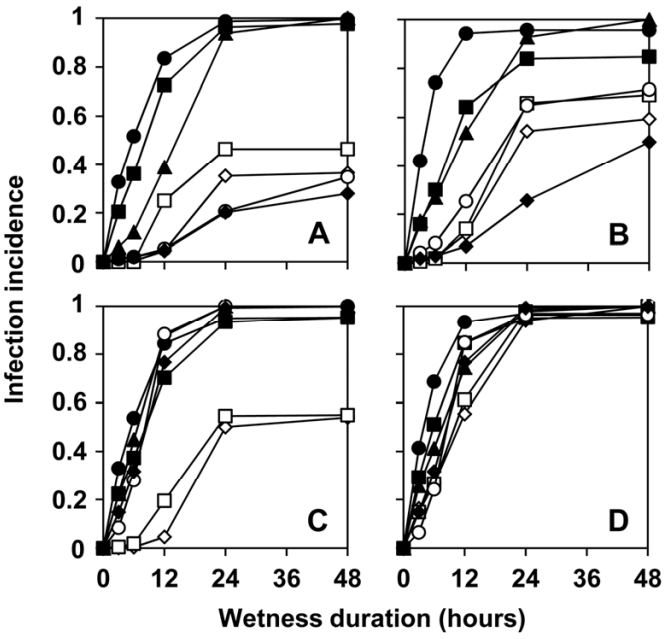

Fig. 5. Infection by four *Botrytis cinerea* strains (**A,** strain 321V; **B,** 18.21V; **C,** 18.13T; and **D,** 213T) following conidial inoculation of grape inflorescences and young berry clusters as affected by host tissue growth stage and hours of wetness at 20°C. Growth stages were ◇ = inflorescence clearly visible (stage 53); □ = inflorescence fully developed (stage 57); ▲ = beginning of flowering, 10% of flowerhoods fallen (stage 61); ● = full flowering, 50% of flowerhoods fallen (stage 65); ■ = end of flowering (stage 69); ○ = fruit set, young fruit begin to swell (stage 71); and ◆ = berries groat-sized (stage 73). Incidence was measured as the proportion of inflorescences or clusters with typical sporulation after 2 weeks at 20°C.

TABLE 2. Area under the infection progress curve (AUIPC) for grape inflorescences and young berry clusters inoculated at seven growth stages with eight *Botrytis cinerea* strains belonging to transposon genotypes *transposa* and *vacuma*

	Growth stage[a]							
	Preflowering		Flowering			Post-flowering		
Genotype, strain	53	57	61	65	69	71	73	Mean[b]
transposa								
18.13T	16.0	14.1	41.5	39.7	37.5	40.6	39.8	32.7
213T	36.5	37.9	39.4	41.5	39.8	38.6	39.8	39.1
344T	29.4	33.1	39.3	42.6	39.4	38.5	37.0	37.1
53T	22.7	35.4	25.9	41.3	37.9	36.9	25.2	32.2
vacuma								
155V	22.5	35.0	37.4	29.5	38.5	30.7	37.3	33.0
18.21V	15.0	17.1	36.3	41.9	33.6	23.5	12.2	25.7
321V	11.6	20.5	34.4	40.4	38.8	8.7	11.0	23.6
351V	34.6	28.0	37.0	40.5	38.9	41.0	38.4	36.9
Mean[b]	23.5	27.7	36.4	39.7	38.0	32.3	30.1	32.5

[a] Growth stages: inflorescence clearly visible (stage 53); inflorescence fully developed (stage 57); beginning of flowering, 10% of flowerhoods fallen (stage 61); full flowering, 50% of flowerhoods fallen (stage 65); end of flowering (stage 69); fruit set, young fruit begin to swell (stage 71); and berries groat-sized (stage 73).

[b] Least square difference (LSD) at P = 0.05. LSD stage = 2.73 and LSD stage–strain (within transposon genotype) = 3.89. AUIPC was not significantly influenced (P > 0.05) by the main effects of fungal transposon genotype or strain within transposon genotype but was significantly influenced (P < 0.001) by inflorescence growth stage at the time of inoculation and the stage–fungal strain (within transposon genotype) interaction.

(Table 2). Because of the interaction between growth stage and fungal strain, the above general pattern was valid for some strains (e.g., 321V and 18.21V) but not for others (e.g., 18.13T and 213T).

For strains 321V and 18.21V, the AUIPC was higher following inoculations at flowering than for the inoculations before and after flowering (Table 2). For the inoculations made at stages 61 to 69 with these strains, the infection incidence was 0.85 to 1 after 48 h of WD (Fig. 5A and B). For the other growth stages, infection incidence ranged from 0.28 to 0.47 for strain 321V (Fig. 5A) and from 0.50 to 0.72 for strain 18.21V (Fig. 5B).

Unlike the AUIPC for strains 321V and 18.21V, the AUIPC for strain 18.13T was approximately 2.5 times lower for inoculations at the preflowering stages 53 and 57 than for all the other stages (Table 2), and infection incidence was also lower at stages 53 and 57 than for other stages (Fig. 5C). The AUIPC for strain 213T was high for inoculations made at every growth stage, showing that this strain was the most virulent among all strains tested (Table 2). For this strain, infection incidence was approximately 1 (i.e., 100% of the grape inflorescences or young berry clusters were infected when inoculated and then kept at 20°C with a WD of 48 h) for all growth stages tested (Fig. 5D). The disease caused by strain 213T progressed substantially more slowly, however, following inoculation of the preflowering stages 53 and 57 than following inoculation of stage 69 (Fig. 5D).

Influence of temperature and WD on infection. Based on the above results, strains 18.13T and 18.21V were selected for studying the effect of temperature and WD on infection incidence on grape inflorescences and young bunches. The optimal temperature for infection by *B. cinerea* conidia was 20°C for all tested combinations of fungal strain–growth stage (Fig. 6). Regardless of strain and grapevine growth stage, the lowest infection incidence usually occurred at 30°C. Infection was also low at 5°C. When inflorescences at the preflowering stage 55 were inoculated, infection incidence was higher with strain 18.13T than with strain 18.21V (Fig. 6A versus B).

With Tmin = 0°C and Tmax = 35°C, model 1 provided a good fit of the observed data on rescaled infection incidence for both strains and for all growth stages at which inflorescences and clusters were inoculated with *B. cinerea* conidia. Values were ≥ 0.88 for R^2, ≥ 0.79 for CCC, ≥ 0.89 for EF, and ≥ 0.97 for W; the MAE ranged from 0.04 to 0.07 and the CRM values showed no substantial deviations from the model predictions (Table 3). The plot of predicted versus observed data did not show systematic deviations for any strain or growth stage (Fig. 7).

Curves fitted for the single strains and growth stages did not significantly differ from each other according to the *F* test. The greatest *F* value (0.53), with $P = 0.78$, was for the comparison between curves corresponding to strain 18.13T and 18.21V inoculated at growth stage 55 (Fig. 6A and B, respectively). Therefore, although the capability of causing infection at stages 55, 65, and 73 differed between the two strains (Fig. 6), the response to temperature and WD was similar for the two strains and the three growth stages. The goodness-of-fit of the overall regression was lower than that of single cases (strain–growth stage) but was still high (Table 3).

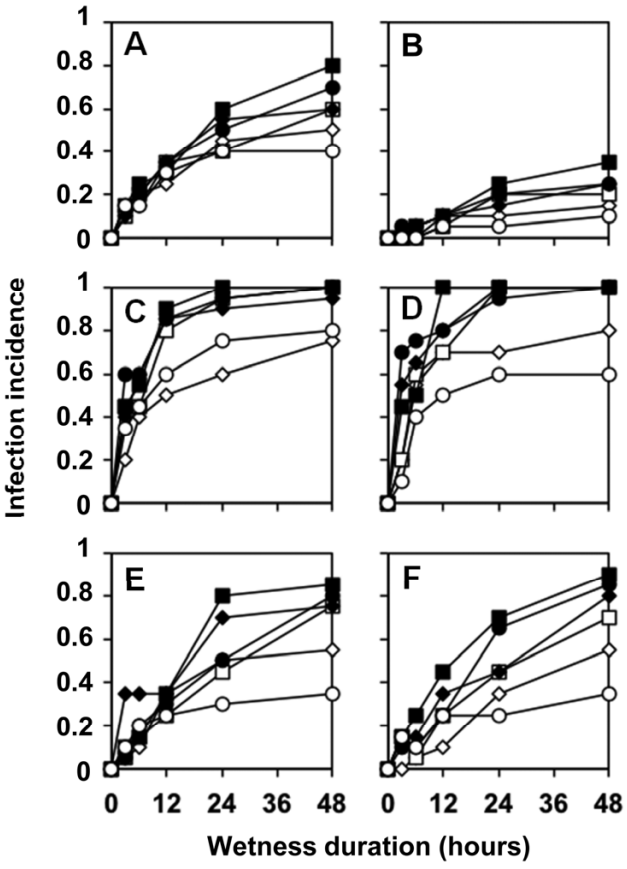

Fig. 6. Infection by two *Botrytis cinerea* strains (**A, C, and E,** 18.13T; **B, D, and F,** 18.21V) following conidial inoculation of grape inflorescences and young berry clusters as affected by temperature, hours of wetness, and three growth stages. Temperature regimes were ◇ = 5°C; □ = 10°C; ● = 15°C; ■ = 20°C; ◆ = 25°C; and ○ = 30°C. Growth stages were inflorescence swelling, flowers closely pressed together (stage 55) (A and B); full flowering, 50% of flowerhoods fallen (stage 65) (C and D); and berries groat-sized (stage 73) (E and F). Incidence was measured as the proportion of inflorescences or clusters with typical sporulation after 2 weeks at 20°C.

DISCUSSION

In the present study, we investigated *B. cinerea* infection of *V. vinifera* inflorescences and young berry clusters at different growth stages. As a general pattern, infection incidence was lowest when inflorescences were developing (stages 53 and 57), highest at the flowering stages (stages 61, 65, and 69), and intermediate at the postflowering stages (stages 71 and 73). The greater infection during flowering may be related to the presence of pollen, which increases conidial germination and germ tube growth (7,11,16,45). The high susceptibility of grapevine flowers may also be caused by their low resveratrol content (39) and is consistent with the high susceptibility to *B. cinerea* of petals in kiwifruit (24), strawberry (8), and rose (78).

Infection assays were performed by using eight *B. cinerea* strains belonging to the transposon genotypes *vacuma* and *transposa*. Infection incidence was mainly influenced by the different strains within each transposon genotype and by the interaction between growth stage and strain. The transposon genotype of a strain accounted for only a small part of the experimental variance (i.e., 6.5%). In other words, the ability of the strains tested in this research to infect grape inflorescences and young berry clusters depended on the particular strain rather than on its transposon genotype. The most virulent strain (strain 213T), however, belonged to the *transposa* transposon genotype.

B. pseudocinerea was not considered in the present study. *B. pseudocinerea* was reported as either absent or occurring at very low frequencies in vineyards (2,22,23,26,27,34,38,41,51,52,71). Thus, *B. pseudocinerea* has been considered of minor epidemiological and agricultural importance (23,64). Similarly, the transposon genotypes *flipper*-only and *boty*-only in *B. cinerea* (former group II of Fournier et al. [27]) were not considered because their frequency in vineyards is low compared with that of *transposa* and *vacuma* genotypes (2,17,28,51,57).

The present study also investigated the effect of temperature and WD on infection by *B. cinerea* and whether this effect is influenced by the strain. For the *transposa* and *vacuma* strains used, infection responses to temperature and WD were similar at any of the inflorescence and berry growth stage tested, with the

highest infection incidence at 20°C and the lowest at 30°C. Infection was also low at 5°C. The results of Nair and Allen (58) were quite different from those obtained here. Nair and Allen inoculated inflorescences before the cap fall stage (stage 61) with conidia of one *B. cinerea* strain that had been isolated from grape flowers (unknown transposon genotype), and they observed the highest infection incidence (96%) after 4 h of wetness at 20°C (58). Under our conditions, 12 h of wetness was necessary to obtain a similar infection incidence at the same growth stage and temperature. Results from our study and Nair and Allen (58) are difficult to compare because of differences in methods. First, we used Barbera while Nair and Allen (58) used 'Cabernet Sauvignon'. Second, we collected inflorescences from a vineyard whereas Nair and Allen (58) used individual flowers from miniaturized plants grown under controlled conditions. Third, we suspended conidia in distilled water containing Tween 20 while Nair and Allen (58) suspended conidia in 0.1% water agar; the use of water agar, in particular, may have favored germination and, thus, enhanced the infection process.

In German vineyards, the optimal temperature for infection by *B. cinerea* was 20°C with at least 16 h of wetness (70). Similarly, in the Bordeaux area of France, Bulit et al. (12) indicated that—both at full flowering and at the end of veraison—a wet period of at least 15 h was required for infection with an average temperature ranging from 15 to 20°C. These findings from Germany and France are in general agreement with the results of the present work.

The present study also investigated the effect of temperature on mycelial growth and the effects of temperature and WD on conidial germination among strains. These effects were quite uniform among the strains used. Both germination and growth occurred between 5 and 35°C, with the optimum at 20°C, regardless of strain. Similar responses have been reported by other authors with single *B. cinerea* strains for both conidial germination (20,29,40,44) and colony growth (9,35,68,74). Martinez et al. (50) also found that both transposon genotypes responded similarly to temperature in terms of colony growth on different media.

Based on these results, we conclude that, regardless of the specific ability of a strain to germinate, grow, and infect grape inflorescences or young berry clusters, the general response to temperature is similar among strains of *B. cinerea*. Therefore, we developed model 1 to account for the combined effect of temperature and WD on relative infection incidence. This equation will be useful for the development of decision-making systems concerning disease control at early grapevine growth stages. In detail, this equation should provide information on when conditions favor *B. cinerea* infection (until fruit set) and predict relative infection incidence. Real disease incidence in a vineyard will also depend on other factors, including the genetic composition of the *B. cinerea* population in the vineyard and its specific ability to cause infection (25,35,54,56,59,72,73). In most cases, Botrytis bunch rot is currently managed by routine applications of synthetic

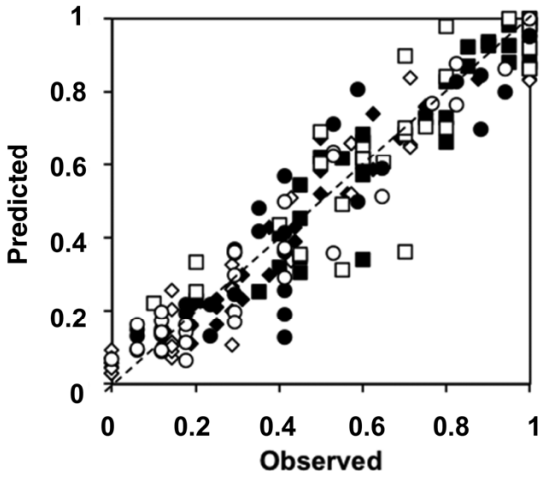

Fig. 7. Predicted versus observed incidence of infection by *Botrytis cinerea*. Predictions were made using model 1 with the parameter estimates in Table 3 for strain 18.13T (dark symbols) and 18.21V (white symbols) and for inoculation of three grapevine growth stages. Growth stages were ◆ and ◇ = inflorescence swelling, flowers closely pressed together (stage 55); ■ and □ = full flowering, 50% of flowerhoods fallen (stage 65); and ● and ○ = berries groat-sized (stage 73). The dashed line shows the perfect agreement between predicted and observed data.

TABLE 3. Parameter estimates, statistics, and indexes of goodness-of-fit of model 1 used for fitting the effect of temperature and wetness duration on the incidence of infection by two *Botrytis cinerea* strains that were used to inoculate grape inflorescences and young berry clusters at three growth stages (GSs)

	Model parameters[b]					Statistic and indexes[c]					
Strain, GS[a]	*a*	*b*	*c*	*d*	*e*	R^2	CCC	EF	W	MAE	CRM
18.13T											
55	2.88	1.00	0.51	1.96	0.17	0.93	0.94	0.95	0.99	0.04	0.03
	0.44	*0.11*	*0.11*	*0.20*	*0.02*						
65	4.35	1.11	0.48	1.75	0.37	0.92	0.86	0.96	0.99	0.05	0.02
	0.45	*0.11*	*0.10*	*0.26*	*0.06*						
73	3.77	0.97	0.73	2.18	0.16	0.88	0.90	0.89	0.97	0.07	0.03
	0.50	*0.12*	*0.18*	*0.30*	*0.03*						
18.21V											
55	3.54	0.98	1.34	2.89	0.17	0.92	0.94	0.94	0.98	0.06	–0.004
	0.25	*0.07*	*0.20*	*0.34*	*0.02*						
65	3.73	0.89	0.63	2.00	0.48	0.89	0.79	0.93	0.98	0.07	0.01
	0.39	*0.10*	*0.13*	*0.40*	*0.09*						
73	4.31	0.98	1.07	2.30	0.13	0.94	0.95	0.95	0.99	0.06	0.02
	0.42	*0.10*	*0.11*	*0.23*	*0.11*						
Overall[d]	3.56	0.99	0.71	1.85	0.19	0.72	0.75	0.75	0.93	0.12	0.03
	0.28	*0.07*	*0.10*	*0.18*	*0.02*						

[a] Growth stages: inflorescence swelling and flowers closely pressed together (stage 55); full flowering, 50% of flowerhoods fallen (stage 65); berries groat-sized (stage 73).

[b] Model 1: $y = [a \times Teq^b \times (1 - Teq)]^c/[1 + \exp(d - e \times x)]$, where y = infection incidence; Teq = equivalents of temperature calculated as (T – Tmin)/(Tmax – Tmin), with T = temperature regime, Tmin = 0°C, and Tmax = 35°C; and x = incubation time in hours of wetness. Numbers in italics = standard error of model parameter estimates.

[c] R^2 = the coefficient of determination, CCC = concordance correlation coefficient, EF = model efficacy EF, W = index of agreement, MAE = mean absolute error, and CRM = coefficient of residual mass.

[d] Both strains and all three growth stages pooled.

fungicides. Treatments are generally recommended at some of the following four key growth stages: end of flowering, prebunch closure, veraison, and before harvest (1,4,10,12,21,43,58). From the stage when the inflorescences begin to be clearly visible until flowering, a fungicide spray may be recommended if environmental conditions are favorable for infection (12,58). Adequate control of infection at such an early growth stage is relevant because infection at these stages may directly result in inflorescence rot (21,35). It may also result in latent infections that become visible as typical Botrytis bunch rot symptoms after veraison (21,31,39,53,62,63,75). Adequate control of infection at the indicated early growth stages is also relevant because infected floral debris may be a source of inoculum within the bunches for later infections (14,21,60,67).

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