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Modeling the Effect of Temperature on the Severity of Blueberry Stem Blight and Dieback with a Focus on *Neofusicoccum parvum* and Cultivar Susceptibility

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Abstract: Stem blight and dieback rank among the most relevant diseases affecting blueberry production worldwide. In Northern Italy, *Neofusicoccum parvum*, *Diaporthe rudis*, *Cadophora luteo-olivacea* and *Peroneutypa scoparia* have been reported to cause stem blight and dieback in blueberry. Considering that the incidence and severity of these diseases are on the rise in Northern Italy, two of the main aims of the present study were a—to compare the in vitro growth rate of the four fungi at different temperatures and b—to assess the aggressiveness of the same fungi on four commercial blueberry cultivars. *Neofusicoccum parvum* had the fastest growth rate and was the most aggressive pathogen. A possible effect of temperature on host colonization by *N. parvum* and disease expression was postulated and tested as a third aim. *In planta* trials were performed to model and predict the influence of temperature on the severity of blueberry stem blight and dieback caused by *N. parvum*. Increasing temperatures boosted the aggressiveness of the pathogen, causing higher disease severity and host mortality. Our findings suggest that temperature plays a relevant role in the severity of blueberry stem blight and dieback caused by *N. parvum.* Given the predictions of a warmer climate, this disease may become increasingly more significant and should be actively managed.

Keywords: epidemiology; generalized analytis beta model; global warming; *Vaccinium corymbosum*

1. Introduction

Stem blight and dieback are common diseases that are seriously compromising the fruit quality and yield of blueberry (*Vaccinium corymbosum)* orchards worldwide [1]. Several fungal species have been reported as putative causal agents of these diseases [2]. The species most frequently isolated from symptomatic blueberry plants belong to the Botryosphaeriaceae family or to the genus *Diaporthe* [2]. *Neofusicoccum arbuti*, *N. australe*, *N. parvum*, *N. nonquaesitum* and *Diaporthe* spp. have been isolated from symptomatic blueberry plants in Chile, often in the same or in contiguous orchards [3]. *Lasiodiplodia* species associated with blueberry dieback have been isolated in Peru [4], while *Botryosphaeria corticis* has been reported in New Jersey and Noth Carolina (USA) [5]. *Neofusicoccum* and *Diplodia* spp. have been detected in farms and nurseries in New Zealand [6], and eight species in the genera *Botryosphaeria*, *Lasiodiplodia* and *Neofusicoccum* have been reported in Australia [7]. *Botryosphaeria dothidea*, *Neofusicoccum parvum* and *Lasiodiplodia theobromae* have been isolated from diseased blueberry plants in China [8], where several new *Lasiodiplodia* species, including *L. vaccinii*, have been identified in association with cankers or wilting of blueberry branches [9]. In Europe, *Diaporthe eres* has been reported in association with dieback,

twig and stem blight and cankers of blueberry in Croatia [10] and the Netherlands [11]. Additionally, *D. viticola* (=*D. rudis*) from the Netherlands and *D. baccae* and *D. sterilis* from Italy are newly described species isolated from symptomatic blueberries [11]. *Neofusicoccum parvum* and *N. australe* have been reported as causal agents of blueberry canker and dieback in Spain [12], while the same diseases in highbush blueberry were related to species of the genus *Lasiodiplodia* in Spain [13] and the Czech Republic [14]. Finally, four Botryosphaeriaceae species (*Botryosphaeria dothidea*, *Neofusicoccum parvum*, *N. australe* and *N. eucalyptorum*) and six *Diaporthe* spp. have been reported as pathogens in blueberry in Portugal [15,16]. In Italy, the adoption of new agricultural practices and cultivars (cvs.), the intensification of cultural systems along with the movement of plant material on a large scale have all contributed to a consistent increase in blueberry production, followed by the emergence of various diseases [17,18]. Recently, stem blight and dieback symptoms have been on the rise and are of growing concern for producers in some areas of Italy. In the northwest of Italy, four different fungal species, namely *Neofusicoccum parvum*, *Diaporthe rudis*, *Cadophora luteo-olivacea* and *Peroneutypa scoparia*, have been confirmed to be pathogens of blueberries, with *N. parvum* and *D. rudis* being the dominant and most virulent ones [18]. It should be noted that Northwestern Italy contributes significantly to the overall Italian blueberry production, with Piedmont including 46.2% of the total blueberry cultivation in the country [19].

Many species in the order Diaporthales and Botryosphaeriales are known for their ability to survive as endophytes or as latent pathogens, until the onsite of conditions favorable to their growth triggers their switch to a pathogenic phase [20,21]. Thus, abiotic factors can play a major role in disease development by affecting plant physiology, which, in turn, may create a favorable environment for latent pathogens. The shift from endophytism to pathogenicity is regulated by the intimate interaction between host physiology and fungal physiology, with each one of these variables, in turn, being strongly influenced by the climate and/or by the presence of other organisms [22,23]. Climate change and global warming are strongly involved in modulating fungal disease incidence and impact woody hosts by directly affecting the fungus and the plant host independently [2,24]. For instance, variations in temperature combined with increasing drought can influence pathogen adaptation, development and spread [25]. Temperature can cause a severe impact on agricultural ecosystems, not only influencing plant physiology, but also the survival, reproduction, transmission and evolution of pathogens [26,27].

Climate change models are predicting a significant rise in summer air temperatures in the Mediterranean area, resulting in drought stress on many fruit crops, which, in turn, may increase their susceptibility [28,29]. Considering the key role of warming temperatures on pathogens, hosts and their interactions, agricultural crops will be likely challenged by global warming in the near future [30]. Disentangling the direct (e.g., directly affecting the pathogen) vs. the indirect (e.g., affecting the pathogen through changes in the host) effects of climate change is a pivotal task for the prediction and management of plant diseases in areas experiencing climate change.

The aims of the present study were as follows:

(i)   To compare the average in vitro growth rate of the four confirmed blueberry stem blight and dieback pathogens in Northwestern Italy at different temperatures (i.e., *Neofusicoccum parvum*, *Diaporthe rudis*, *Cadophora luteo-olivacea* and *Peroneutypa scoparia*);

(ii) To evaluate the susceptibility of different northern highbush blueberry cvs. to these four pathogens;

(iii) To model the in vitro effect of temperature on the mycelial growth rate of *Neofusicoccum parvum*;

(iv) To assess and model the *in planta* effect of temperature on the severity of blueberry stem blight and dieback caused by *Neofusicoccum parvum*, the most virulent of the four pathogens studied here.

2. Materials and Methods

2.1. Fungal Species and Strain Selection

The fungal species investigated here were *Neofusicoccum parvum*, *Diaporthe rudis*, *Cadophora luteo-olivacea* and *Peroneutypa scoparia*. All four are confirmed pathogens of blueberry [18]. One representative strain of each fungal species (CVG444—*N. parvum*, CVG561—*P. scoparia*, CVG651—*C. luteo-olivacea*, CVG658—*D. rudis*) was selected for this study. The identity of each strain was confirmed thanks to a multi locus phylogenetic analysis based on the nuclear ribosomal internal transcribed spacer (ITS), the β-tubulin (*tub2*) and translation elongation factor-1α (*tef1*). All strains were collected from wilted blueberry twigs and cankered branches in 2019 and kept at −80 °C in the University of Torino culture collection area, Torino, Italy. Their pathogenicity was previously assessed thanks to inoculations performed on blueberry plants of the cv. Duke [18]. The most aggressive species in the pathogenicity study was *N. parvum* [18]. Fungal strains were grown on potato dextrose agar (PDA, VWR Chemicals, Leuven, Belgium) amended with 25 mg/L of streptomycin sulphate (PDA-S, AppliChem GmbH, Darmstadt, Germany) at 25 ± 1 °C for 7 days in the dark. Mycelial plugs were cut with a cork borer (4 mm diam) from the margin of actively grown colonies and used for the inoculation of plates and plants.

2.2. Plant Material

The northern highbush blueberry cvs. 'Blue Ribbon', 'Cargo', 'Last Call' and 'Top Shelf' were selected to compare the virulence of the four fungal strains. The cvs. Blue Ribbon, Last Call and Top Shelf were selected because the fungal strains used in this study were originally isolated from such cultivars. 'Cargo' was included due to its increased popularity associated with the demand for varieties with different ripening times [31]. Plants of the cv. Duke were additionally used to assess the *in planta* effect of temperature on the colonization rate and branch mortality caused by *N. parvum*. This cv. was selected for the *in planta* portion of the study given that it is currently the most widely cultivated worldwide.

2.3. Comparison of In Vitro Growth Rates of Cadophora luteo-olivacea, Diaporthe rudis, Neofusicoccum parvum and Peroneutypa scoparia

Mycelial plugs (6 mm diam.) of the four selected strains were placed onto the center of new PDA-S plates and incubated at 5, 10, 15, 20, 25, 30 and 35 °C in the dark. Seven replicate Petri dishes per strain and temperature combinations were considered in a completely randomized design. Two perpendicular diameters of the colonies were measured with a scale ruler once daily for 4 to 7 days post inoculation. Mean data were converted to radial growth rate (mm/day). Average in vitro growth rates (IGR, mm/day) were calculated for each of the four fungal species along with their 95% bias-corrected and accelerated confidence intervals ($_{95\%BCa}$CI) [32]. Bounds of confidence intervals were obtained from 10^4 bootstrap resamplings as described in the study by Lione et al. [33]. Average IGRs were compared among fungal species by fitting an unbiased recursive partitioning tree model based on conditional inference [34,35], whose algorithm was initialized as reported by Lione et al. [36].

2.4. Varietal Susceptibility Test

Potted 1-year-old healthy northern highbush blueberry plants were used. Seven 'Blue Ribbon', 'Cargo' and 'Top Shelf' plants and five 'Last Call' plants were considered for each selected fungal strain to conduct the inoculation trials. Each plant was inoculated on four branches with a single fungal strain. The inoculation point of each branch was surface disinfected with 70% ethanol solution. A piece of bark was cut with a sterile scalpel to expose the cambium. Mycelium-colonized agar plugs (4 mm diam.) were placed on the wound with the mycelium in contact with the cambium. Each inoculation point was wrapped with Parafilm® (American National Can, Chicago, IL, USA). The same number of plants per cv. and branches per plant was treated with sterile PDA-S discs as inoculation

controls. The plants were placed in a growth chamber at 24 °C with 12 h light per day and monitored daily for external necrosis and dead branch development. Mortality of branches was assessed at two and four weeks post inoculation. After seven weeks, a destructive assessment was conducted. The Parafilm® was removed, and the external lesion length was measured. Moreover, the internal lesion length was recorded after bark removal. Small pieces (5 mm) of symptomatic tissue from the margins of the lesions were plated onto PDA to re-isolate the inoculated fungal species to fulfil Koch's postulates. Pathogen identity was confirmed through morphology and DNA sequencing of partial regions of the β-tubulin (*tub2*) locus for *N. parvum*, *D. rudis* and *P. scoparia* and of the translation elongation factor-1α (*tef1*) locus for *C. luteo-olivacea*. Varietal susceptibility of blueberry cultivars to the pathogens was assessed by fitting three different unbiased recursive partitioning tree models [34–36]. Models differed for the selected response variable, namely the length of the external or internal necrosis measured for each inoculated plant (quantitative continuous variables, in mm) or the mortality scored at the end of the trial (binary categorical variable set to 1 or 0 for the living or dead plants, respectively). All models included three categorical input variables, with each one characterized by a different number of levels [37]. Input variables were as follows:

- Inoculation treatment (2 levels: "plants inoculated with isolates of the target fungal pathogens" and "control plants mock-inoculated with plugs of sterile PDA-S");
- Blueberry cultivar (4 levels: 'Blue Ribbon', 'Cargo', 'Last Call' and 'Top Shelf');
- Target pathogen species (5 levels: "*C. luteo-olivacea*", "*D. rudis*", "*N. parvum*", "*P. scoparia*" and "none" for control plants).

By applying the *p*-value criterion to the *c*-statistics with a significance threshold set to 5%, tree models were used to detect significant input variables and to define clusters of blueberry cultivars based on their susceptibility to the pathogens [38–40]. Hence, each terminal node of the tree model graphs included a cluster of blueberry cultivars equally susceptible to one, or more, of the inoculated fungal pathogens ($p > 0.05$). Conversely, different terminal nodes hosted clusters of cultivars displaying different levels of susceptibility ($p < 0.05$) to the above pathogens. The unique combination of input variable levels leading to any single terminal node of the tree model was obtained by (I) identifying the paths connecting the root, intermediate and terminal nodes of the graph, accounting for all significant splits ($p < 0.05$); (II) listing the categorical variable levels associated with the sequence of edges connecting nodes along the path; and (III) removing the redundant duplicates of the same level from the list [35,41]. For plants clustering within each terminal node, the average of external and internal necrosis was calculated, along with the corresponding $_{95\%BCa}CI$. The ratio between dead plants and total number of plants (mortality rate, in %) was calculated with its associated 95% exact confidence interval ($_{95\%E}CI$) for terminal nodes of the mortality tree model. Upper and lower bounds of the confidence interval were derived as described by Blaker [42].

The fungus *N. parvum* was selected for the *in planta* temperature trial given its greatest growth rate in vitro and its highest pathogenicity *in planta*.

2.5. Modeling the Effect of Temperature on the Mycelial Growth Rate of Neofusicoccum parvum In Vitro

The mycelial growth rate of *Neofusicoccum parvum* was modeled as a function of temperature by fitting the generalized Analytis Beta Model to data obtained in vitro through the following equations [43]:

$$Y = d \cdot (T - T_{min})^a \cdot (T_{max} - T)^b \tag{1}$$

$$T_{opt} = \frac{a \cdot T_{max} + b \cdot T_{min}}{(a + b)} \tag{2}$$

In Equation (1), Y is the radial growth rate of the fungal colonies (mm/day), T_{min} and T_{max} are the minimum and maximum growth temperatures set to conduct the experiment

(i.e., 5 and 35 °C, respectively) and T is the temperature level (°C) of each replication of the trial (i.e., 5, 10, 15, 20, 25, 30 and 35 °C). In Equation (2), T_{opt} is the optimum temperature, corresponding to the maximal mycelial growth rate of *N. parvum* in vitro. For both equations, a, b and d are unknown parameters. Equation (1) was fitted to data through nonlinear regression based on the Levenberg–Marquardt algorithm [44–46]. The algorithm was initialized by setting the starting values at a = 2, b = 2 and d = 2 using the data reported in the study by López-Moral et al. [43] as reference. The performance of the generalized Analytis Beta Model resulting from the algorithm convergence was assessed as described by Lione et al. [39] by fitting a linear regression model between the observed and predicted growth rate values (OP-regression) [47] and calculating the Theil's UII coefficient [48].

2.6. In Planta Effect of Temperature on Colonization Rate and Branch Mortality of N. parvum

Based on the results obtained from the in vitro assay, temperatures of 18, 22, 26 and 30 °C were selected to assess the *in planta* effect of temperature on the colonization rate and branch mortality of *N. parvum*. Strain CVG444 of *Neofusicoccum parvum* was inoculated into potted 1-year-old healthy northern highbush blueberry plants of the cv. Duke. Inoculations were performed as described above on one branch of each of the thirty plants per temperature. Twenty plants per each temperature were mock-inoculated using sterile PDA-S discs. Plants were incubated in four different growth chambers and constantly monitored for symptoms and signs of development. After two weeks, a destructive assessment was conducted due to the high visual presence of symptoms. Parafilm® was removed, plants were carefully inspected for signs of the pathogen and external lesion lengths were measured. Internal lesion lengths were measured after bark removal. Branches' mortality was recorded.

2.7. Modeling the Effect of Temperature on the Severity of Neofusicoccum parvum in Planta

To model the effects of temperature on the severity of *N. parvum in planta*, the data obtained from the inoculation trials were analyzed by using the same unbiased recursive partitioning tree models and response variables reported in the section describing the blueberry varietal susceptibility test. Categorical input variables were set as follows:

- Inoculation treatment (2 levels: "plants inoculated with the selected isolate of *N. parvum*" and "control plants mock-inoculated with plugs of sterile PDA-S");
- Temperature (4 levels: "18 °C", "22 °C", "27 °C" and "30 °C").

For each blueberry plant, the average increase in the necrosis severity (AINS, mm/day) was calculated as the ratio between the internal or external necrosis lengths at the end of the trial and the number of days since inoculation. AINS values were transformed into a series of binary variables by applying a threshold (X), with levels coded as 1 for AINS > X, as 0 for AINS ≤ X [49]. Thresholds were set based on increasing severity levels at X = 2, X = 5, X = 10, X = 15 and X = 20 mm/days. Hence, binary logistic regressions [49] were fitted to model the probability of observing AINS values larger than the above threshold as a function of increasing temperatures. The fulfillment of the models' adequacy conditions was assessed as reported by Lione et al. [36] through a—the application of the Wald's test to the β and $β_0$ coefficients [49,50]; b—the comparison of the fitted model to the corresponding null-model M_0, assessing the overall regression significance with the likelihood ratio test [49,50]; and, c—the comparison of the Akaike information criterion (AIC) and AIC weight (AICw) values between the non-null and the null models [51,52]. Mortality rates were calculated along with their 95%ECI values and analyzed as described by Garbelotto et al. [53] by using the Fisher's Exact test to compare the blueberry plants inoculated with the selected isolate of *N. parvum* and the control plants at each temperature level. A binary logistic regression equation was fitted and assessed as previously described to model the probability of death of the inoculated plants as a function of temperature.
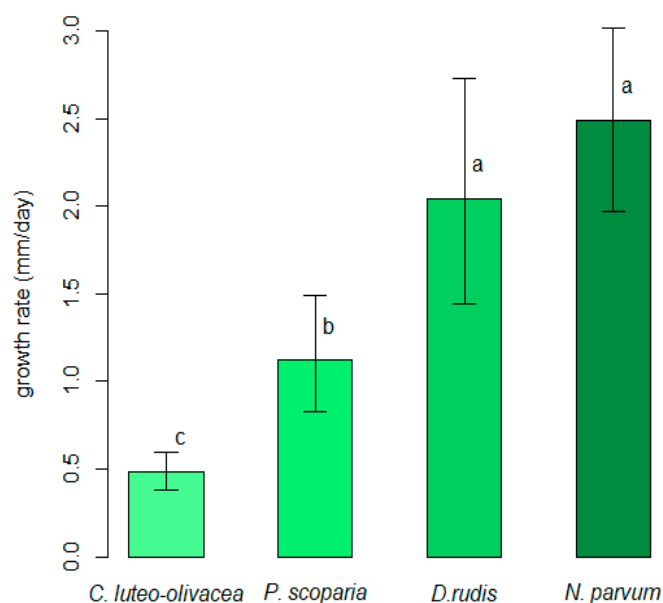
3. Results

3.1. Comparison of In Vitro Growth Rates of Cadophora luteo-olivacea, Diaporthe rudis, Neofusicoccum parvum and Peroneutypa scoparia

All of the strains were able to grow on PDA-S in the 10 to 30 °C range of temperatures. Weak mycelial growth was observed at 5 °C for the *C. luteo-olivacea* and *D. rudis* strains, while *N. parvum* and *P. scoparia* showed weak mycelial growth at 35 °C. The unbiased recursive partitioning tree model showed that *N. parvum* was the fungal species that displayed the highest average values regarding the in vitro growth rate (IGV = 2.49 mm/day, 1.97–3.01 $_{95\%BCa}$CI), followed by *D. rudis* (2.04 mm/day, 1.44–2.73 $_{95\%BCa}$CI), *P. scoparia* (1.12 mm/day, 0.83–1.49 $_{95\%BCa}$CI) and *C. luteo-olivacea* (0.49 mm/day, 0.38–0.59 $_{95\%BCa}$CI), respectively. The difference detected among the average IGRs were significant ($p < 0.05$) for all fungal species, except for that between *N. parvum* and *D. rudis* (Figure 1).

Figure 1. Comparison of average in vitro growth rates among *Cadophora luteo-olivacea*, *Diaporthe rudis*, *Neofusicoccum parvum* and *Peroneutypa scoparia*. Different letters indicate that associated values are different ($p < 0.05$) based on outcomes of unbiased recursive partitioning tree model. Error whiskers refer to 95% bias-corrected and accelerated confidence interval bounds.

3.2. Varietal Susceptibility Test

The four pathogens caused different levels of disease severity when inoculated on the four cvs. of northern highbush blueberry. The analyses grouped pathogens and cultivars with similar interactions based on decreasing levels of the observed symptoms severity. The unbiased recursive partitioning tree model employing the length of the external necrosis as a response variable resulted in a graph leading to eight clusters (i.e., terminal nodes) derived from seven significant splits ($p < 0.05$) associated with all input variables (i.e., blueberry cultivar, inoculation treatment and fungal species) (Figure S1). The susceptibility levels based on the external necrosis length were variable, depending on the combination of fungal species inoculated and on the host blueberry cultivar. The cluster displaying the highest severity level ($p < 0.05$) grouped all blueberry plants inoculated with *N. parvum*, regardless of the cultivar. The average length of the external necrosis caused by *N. parvum* was 25.17 mm (21.45–30.22 $_{95\%BCa}$CI), while the other clusters, including other fungi, had an average external necrosis below 8.20 mm (7.40–8.85 $_{95\%BCa}$ CI). All fungal pathogens inoculated in blueberry plants induced necroses greater ($p < 0.05$) than those in the mock-inoculated plants, with the exception of *D. rudis* on 'Last Call' (Figure 2).
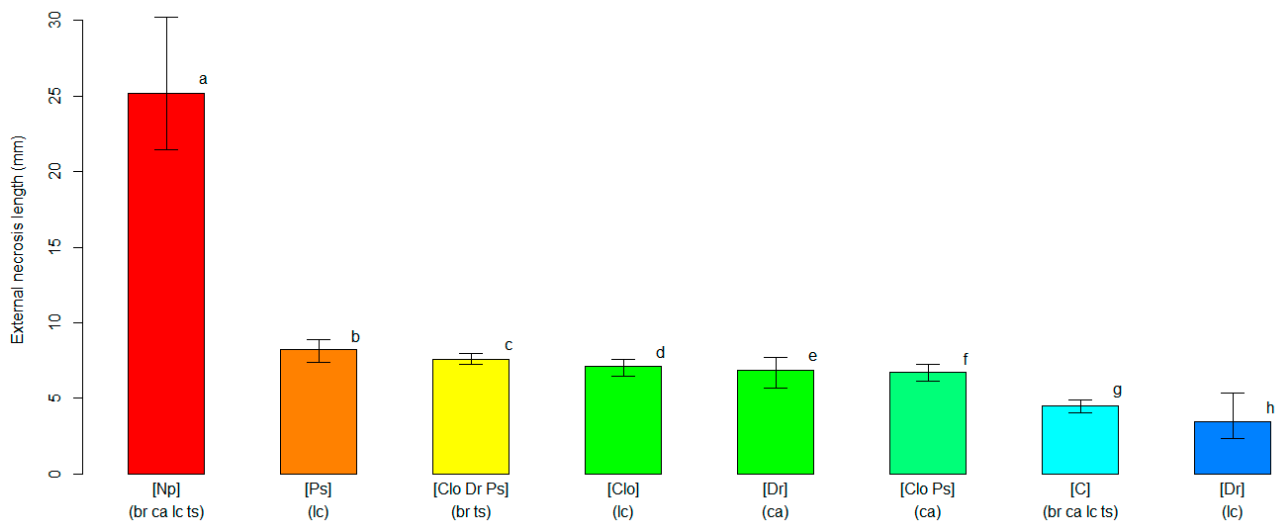
Figure 2. A comparison of the average length of the external necrosis (in mm, on the *y*-axis) among clusters of blueberry plants identified by the unbiased recursive partitioning tree model. Each bar represents a cluster of blueberry cultivars (listed within round brackets) that are equally susceptible (*p* > 0.05) to one or more of the inoculated fungal pathogens [listed within squared brackets]. The cultivar acronyms are defined as follows: br, 'Blue Ribbon'; ca, 'Cargo'; lc, 'Last Call' and ts, 'Top Shelf'. The pathogens' acronyms are Clo, *Cadophora luteo-olivacea*; Dr, *Diaporthe rudis*; Np, *Neofusicoccum parvum* and Ps, *Peroneutypa scoparia*. The acronym C represents control plants that were mock-inoculated with plugs of sterile agar medium. Different letters indicate that the associated cluster bars display significantly different average values of the external necrosis length (*p* < 0.05). The error whiskers refer to the 95% bias-corrected and accelerated confidence interval bounds.

Similar results were obtained from the unbiased recursive partitioning tree model employing the length of the internal necrosis as a response variable (Figure S2). The tree model graph showed nine significant clusters (*p* < 0.05), and the highest severity score (*p* < 0.05) was found for plants inoculated with *N. parvum*, with an average length of the internal necrosis equaling 28.61 mm (24.11–33.45 $_{95\%BCa}$CI). As previously observed for the analysis of the external necrosis, all plants of the four blueberry cultivars inoculated with *N. parvum* were grouped within the same cluster, displaying comparable levels of susceptibility to the pathogen (*p* > 0.05). Internal necroses by the other pathogens were variable, and the average values ranged between 0.96 mm (0.43–1.68 $_{95\%BCa}$CI) for 'Cargo' plants inoculated with *P. scoparia* and 11.58 mm (10.50–12.78 $_{95\%BC}$aCI) for the cluster grouping 'Blue Ribbon' and 'Top Shelf' plants inoculated with *C. luteo-olivacea* and *D. rudis*. Hence, *C. luteo-olivacea*, *D. rudis* and *P. scoparia* induced internal necrosis with average lengths that were 2.5- to 30-fold lower than those caused by *N. parvum* (Figure 3). All average internal necrosis lengths were significantly larger (*p* < 0.05) on blueberry plants inoculated with the pathogens than on the mock-inoculated controls, with the exception of the 'Cargo' plants inoculated with *P. scoparia*.

The unbiased recursive partitioning tree model analyzing mortality rates showed that only the inoculation treatment and inoculated fungal species were significant explanatory variables (*p* < 0.05), while the blueberry cultivar did not have any significant effect on plant mortality (*p* > 0.05) (Figure S3). Regardless of the cultivar, blueberry plants inoculated with *N. parvum* reached an average mortality rate of 82.7% (74.2–89.3 $_{95\%E}$CI), while the control plants and plants inoculated with the other pathogens attained a lower value (*p* < 0.05) of 4.0% (2.3–6.2 $_{95\%E}$CI) (Figure 4). All of the inoculated fungi were re-isolated from the plants on which they had been inoculated, and their identities were confirmed through morphology and molecular features, thus fulfilling Koch's postulates.
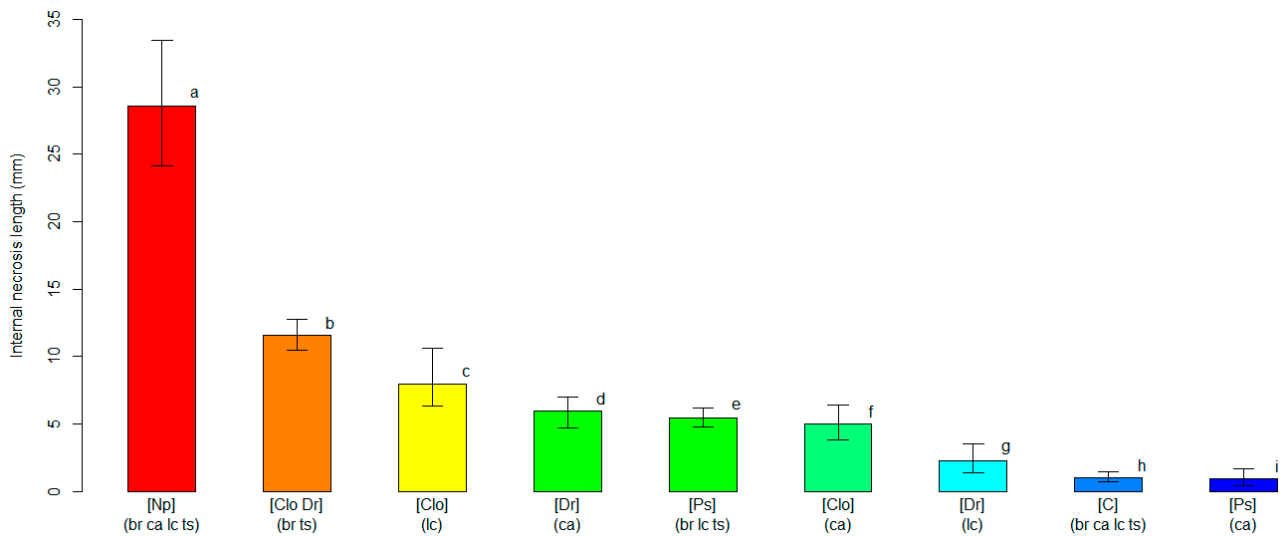
Figure 3. A comparison of the average length of the internal necrosis (in mm, on the *y*-axis) among clusters of blueberry plants identified by the unbiased recursive partitioning tree model. Each bar represents a cluster of blueberry cultivars (listed within round brackets) that are equally susceptible ($p > 0.05$) to one or more of the inoculated fungal pathogens [listed within squared brackets]. The cultivar acronyms are defined as follows: br, 'Blue Ribbon'; ca, 'Cargo'; lc, 'Last Call' and ts, 'Top Shelf'. The pathogens' acronyms are Clo, *Cadophora luteo-olivacea*; Dr, *Diaporthe rudis*; Np, *Neofusicoccum parvum* and Ps, *Peroneutypa scoparia*. The acronym C represents control plants that were mock-inoculated with plugs of sterile agar medium. Different letters indicate that the associated cluster bars display significantly different average values of the internal necrosis length ($p < 0.05$). The error whiskers refer to the 95% bias-corrected and accelerated confidence interval bounds.
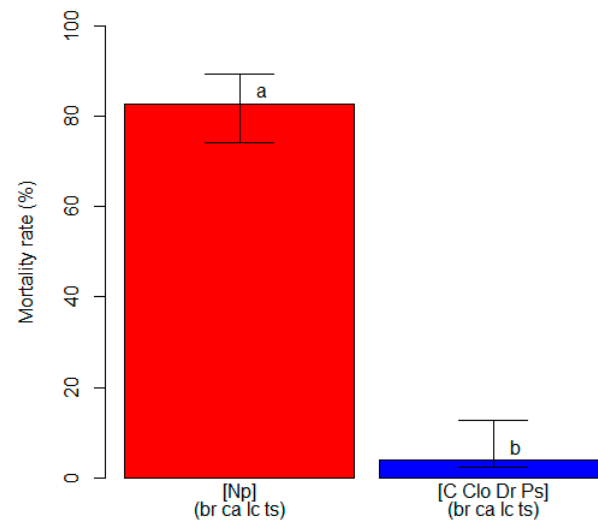

Figure 4. A comparison of the mortality rate (in %, on the y-axis) among clusters of blueberry plants identified by the unbiased recursive partitioning tree model. Each bar represents a cluster of blueberry cultivars (listed within round brackets) that are equally susceptible ($p > 0.05$) to one or more of the inoculated fungal pathogens [listed within squared brackets]. The cultivar acronyms are defined as follows: br, 'Blue Ribbon'; ca, 'Cargo'; lc, 'Last Call' and ts, 'Top Shelf'. The pathogens acronyms are Clo, *Cadophora luteo-olivacea*; Dr, *Diaporthe rudis*; Np, *Neofusicoccum parvum* and Ps, *Peroneutypa scoparia*. The acronym C represents control plants that were mock-inoculated with plugs of sterile agar medium. Different letters indicate that the associated cluster bars display significantly different mortality rate values ($p < 0.05$). The error whiskers refer to the 95% exact confidence interval bounds.

3.3. Modeling the Effect of Temperature on the Mycelial Growth Rate of Neofusicoccum parvum In Vitro
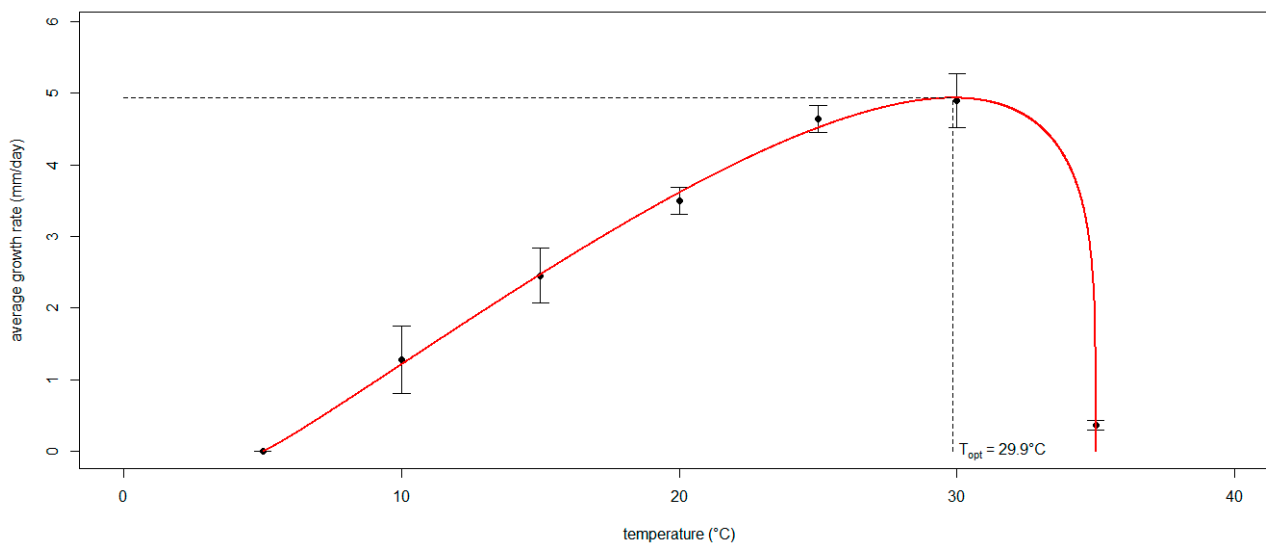
The algorithm run to define the three coefficients of the generalized Analytis Beta Model expressing the mycelial growth rate of *Neofusicoccum parvum* as a function of temperature converged on values that are significantly different from 0 ($p < 0.05$): a = 1.096, b = 0.226 and d = 0.100 (Figure 5). The resulting optimal temperature was 29.9 °C, corresponding to a maximum radial growth rate of approximately 5 mm/day. The model performance was scored as adequate since the OP regression slope (m = 0.967) and intercept (q = 0.129) were not significantly different from 1 and 0, respectively ($p > 0.05$); $R^2 = 0.974$ was close to 1, and Theil's coefficient (UII = 0.0997) was close to 0.

Figure 5. The curve of the generalized Analytis Beta Model expressing the mycelial growth rate of *Neofusicoccum parvum* as a function of temperature. The black dots interpolated by the curve (in red) show the average radial growth rate displayed by the isolates grown in vitro at different temperatures. The whiskers indicate the standard deviation. The dashed lines mark the optimal temperature with the corresponding peak of the radial growth rate.

3.4. Modeling the Effect of Temperature on the Severity of Neofusicoccum parvum in Planta

Neofusicoccum parvum induced different levels of disease severity on the inoculated plants depending on the temperature. The unbiased recursive partitioning tree models showed that both internal (Figure S4) and external (Figure S5) necrosis induced by *N. parvum* on plants of blueberry displayed average lengths that were larger ($p < 0.05$) than those of the control plants that were mock-inoculated with plugs of sterile agar medium (Figure 6). The above difference remained significant ($p < 0.05$) at all temperatures employed in this study. The average lengths of necrosis among the control plants were not significantly different ($p > 0.05$) regardless of the temperature and type of necrosis considered. The average lesion lengths in the controls ranged between 0.40 mm (0.10–0.80 95%BCaCI) and 1.30 mm (0.70–2.20 95%BCaCI) for internal necrosis and between 0.20 mm (0.00–0.30 95%BCaCI) and 1.60 mm (0.60–2.70 95%BCaCI) for the external one.

Conversely, on average, both the internal and external necrosis lengths increased ($p < 0.05$) with the increasing temperature levels. For instance, the inoculations of *N. parvum* conducted at 30 °C led to an average internal necrosis length of 278.00 mm (235.30–323.80 95%BCaCI), which is higher ($p < 0.05$) than that found in blueberry plants inoculated at 18 °C (87.60 mm, 76.00–98.10 95%BCaCI). Similarly, while at 18 °C, the average external necrosis length was 32.00 mm (25.50–41.50 95%BCaCI), the blueberry plants inoculated at 30 °C developed an external lesion with an average length of 230.70 mm (190.00–276.70 95%BCaCI).
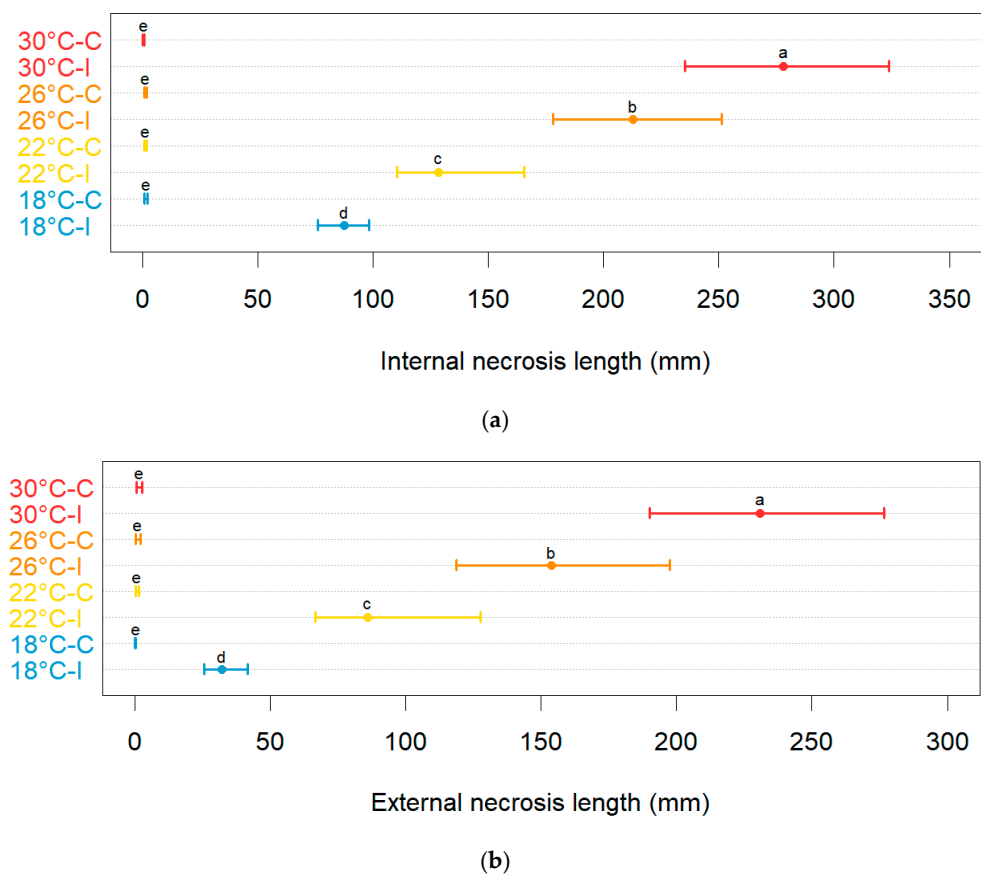
(a)

(b)

Figure 6. Comparisons of the average lengths of internal (**a**) and external (**b**) necrosis (in mm, on the *x*-axis) among the blueberry plants inoculated with *Neofusicoccum parvum* (I) and the control plants (C) that were mock-inoculated with plugs of sterile agar medium at different temperature levels (in °C, on the *y*-axis). Different letters in the dot chart indicate that the associated averages are significantly different ($p < 0.05$). The error whiskers refer to the 95% bias-corrected and accelerated confidence interval bounds.

Consistent results were obtained from the binary logistic regressions modeling the average increase in the necrosis severity (AINS). In brief, the logistic curves showed that the necrosis caused by *N. parvum* grew significantly faster with warming temperatures ($p < 0.05$) (Figure 7). In fact, rising temperatures led to a higher frequency of symptoms of a given severity level, as evidenced by the ascending trend of each logistic curve. Moreover, increasing temperatures caused more severe symptoms, as evidenced by the left-to-right shift in the curves associated with augmented threshold levels (i.e., shifting from blue to red in Figure 7). Technically, all logistic equations, except one, displayed a—a significant and positive β coefficient for temperature ($p < 0.05$); b—a significant overall likelihood ratio test ($p < 0.05$); and c—lower AIC and higher AIC_W values than each associated null model, thus fulfilling the whole set of adequacy conditions. Details on regressions modeling the AINS calculated on the internal and external necrosis lengths are reported in Tables S1 and S2, respectively.

The analysis of the mortality rates showed that while there was no mortality in the control plants, regardless of the temperature level, a variable proportion of dead plants was present among those inoculated with *N. parvum* (Figure 8).
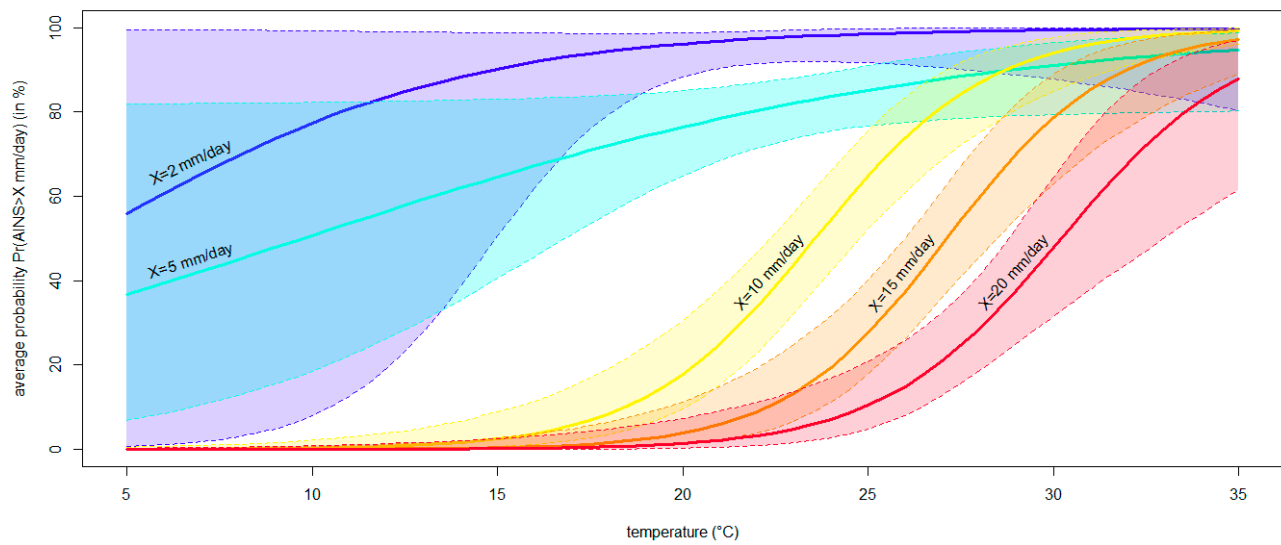
(**a**)
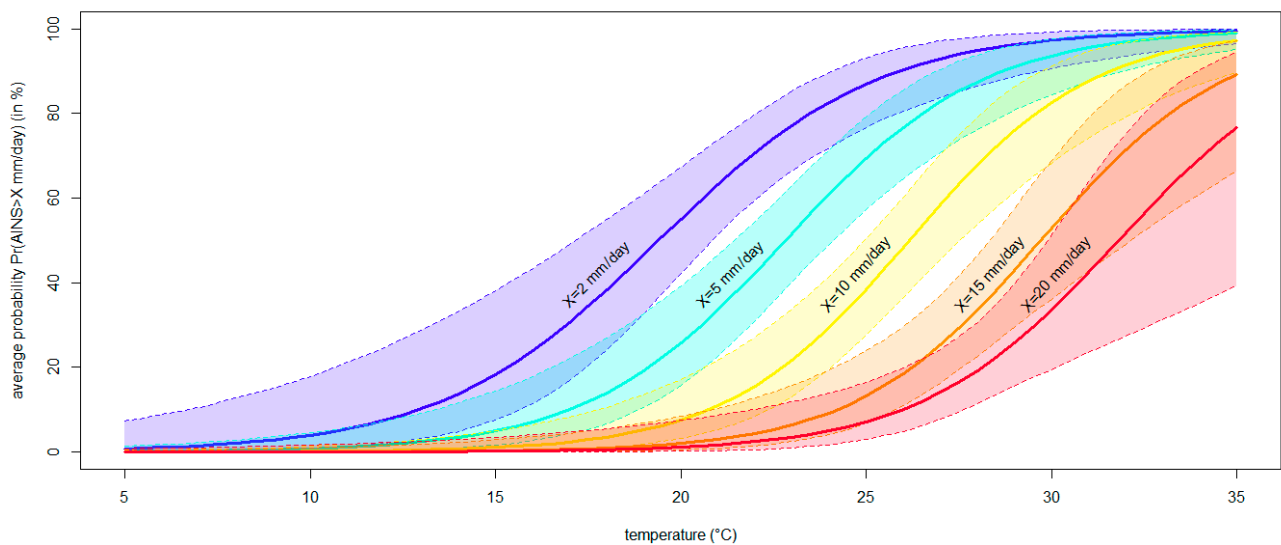
(**b**)

Figure 7. The binary logistic regression curves modeling the probability (Pr, in %) of observing an average increase in the necrosis severity (AINS, mm/day) larger than a given threshold X (set to 2, 5, 10, 15 and X = 20 mm/days) at different temperatures (°C). Curves resulting from the models fitted to AINS calculated from internal (**a**) or external (**b**) necrosis lengths are shown. The region between the contours above and below the curves delimits the 95% confidence interval of the modeled probability.

The percentage of mortality increased with increasing temperature levels, ranging from 0% (0–10.6 $_{95\%E}$CI) at 18 °C to 36.7% (20.9–55.1 $_{95\%E}$CI) at 30 °C, although the Fisher's Exact test comparing the mortality rates was only significant ($p < 0.05$) at the highest temperature. A significant binary logistic regression model ($p < 0.05$) showed that the probability of death in blueberry plants inoculated with *N. parvum* rose with increasing temperature levels (Figure 9). The model fulfilled the adequacy conditions required (Table S3).
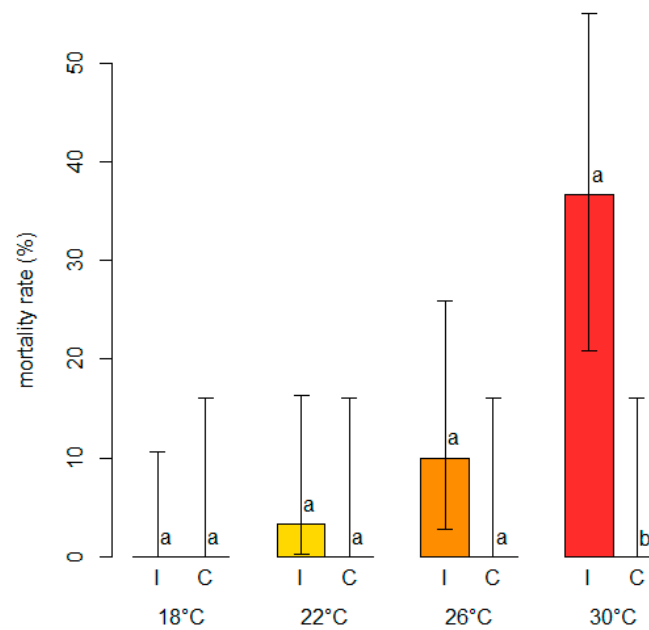
Figure 8. Comparisons of mortality rates (in %, on the *y*-axis) among blueberry plants inoculated with *Neofusicoccum parvum* (I) and control plants (C) mock-inoculated with plugs of sterile agar medium at different temperature levels (°C). Different letters in the bar chart indicate that the associated averages are significantly different ($p < 0.05$). The error whiskers refer to the 95% exact confidence interval bounds.
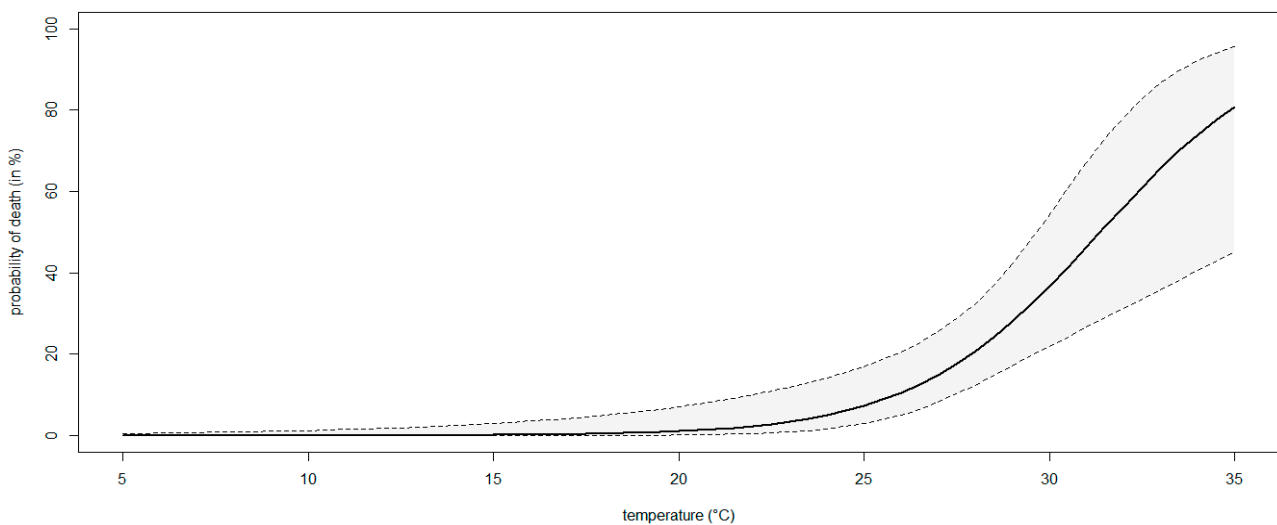

Figure 9. A binary logistic regression curve modeling the probability of death (%) of blueberry plants inoculated with *Neofusicoccum parvum* at different temperatures (°C). The region between the contours above and below the curve delimits the 95% confidence interval of the modeled probability.

4. Discussion

Global warming represents one of the most important and difficult challenges for agriculture [54]. An elevated air temperature is a serious problem for several crops, as it affects the physiology of plants and may enhance their susceptibility to pathogens [25]. Moreover, rising temperatures influence the development of fungal pathogens, modulating the expression of toxic compounds, influencing the infection processes and favoring spore germination and mycelial growth, thus resulting in the emergence of diseases caused by thermophilic fungi [26,55]. In the present study, we first assessed the in vitro effect of temperature on the mycelial growth of four pathogens causing blueberry stem blight

and dieback. The experiment showed that mycelial growth was variable depending on the species. The comparison of in vitro growth rates among the four target pathogens suggests that *N. parvum* is the species which colonizes host plant tissues at the fastest rate. Nonetheless, the growth rate of *D. rudis* was statistically undistinguishable from that of *N. parvum*. Species within the Botryosphaeriaceae are well known to be fast-growing [56], while *Diaporthe rudis* is described as relatively slow-growing [57], so our results regarding *D. rudis* are somewhat unexpected. Further research is needed to confirm that faster growth rates among Botryosphaeriales and Diaporthales always equate to greater disease severity.

The varietal susceptibility test showed that disease severity, expressed as the length of necroses, was highest when the plants were inoculated with *N. parvum*. At the same time, *N. parvum* caused the highest mortality rates regardless of the blueberry cultivar. Conversely, not only did the other tested pathogens display lower levels of virulence, but virulence varied depending on the cultivar. In spite of its good growth *in vitro*, *D. rudis* displayed mild virulence *in planta*, causing lesions that, in some cases, were undistinguishable from those observed in the control plants. The superiority of *N. parvum* as a pathogen was well exemplified by the clustering of mortality rates, which indicated that the proportion of dead plants was approximately 20-fold higher when the plants were inoculated with *N. parvum* than the proportion of dead plants that were inoculated with the other three fungi or mock-inoculated. All of the above results strongly support the selection of *N. parvum* as an ideal species to test and model the effects of increasing temperature levels on the growth rate and virulence of a fungal plant pathogen.

The generalized Analytis Beta Model resulted in an inverse U-shaped and highly asymmetric curve, displaying a mildly increasing pattern of growth rates between 5 °C and 29.9 °C (optimal temperature), followed by a steep drop in growth between 29.9 °C and 35 °C. Based on this theoretical model, any positive discrete variation in temperature ($\Delta t > 0$) occurring between 5 °C and $29.9 - \Delta t$ °C leads to a corresponding positive increment in the substrate colonization ability of *N. parvum* ($\Delta c > 0$). If this model developed in vitro held true *in planta*, it might be argued that the severity of the symptoms observed in the host plants infected by *N. parvum* should increase with increasing temperature levels. This hypothesis was strongly supported by the results of the inoculations of *N. parvum* in plants of blueberry grown in an environment where the temperature was controlled. In these trials, the air temperature increased by $\Delta t = 4$ °C, starting at 18 °C and ending at 30 °C, leading to a corresponding significant and positive increment in symptom severity (i.e., internal and external necrosis lengths). Probabilistic models based on binary logistic regression not only show that warming temperatures are positively correlated with a higher probability of observing larger lesions, but also that lesion sizes at higher temperatures may be substantially larger. Similarly, the mortality rates of blueberry plants inoculated with *N. parvum* also increased with increasing temperatures. Interestingly, but not surprisingly, the peak of mortality was observed at 30 °C, which corresponds to the optimal temperature for the mycelial growth of *N. parvum* in the Analytis Beta Model developed from in vitro observations. The logistic equations predict that warming temperatures lead to an increasing probability of death in plants infected by *N. parvum*. This probability calculation may be a proxy to estimate yield losses in different climate change scenarios. Incidentally, the pathogen was also able to produce visible oozing pycnidia on plants at 30 °C, which is in line with what was reported in previous studies conducted on blueberry and grapevine [58,59]. This aspect suggests that high temperatures, even for a prolonged period, may not only increase disease severity in the field, but may also lead to sporulation, thus providing an additional inoculum source for further infections.

Based on the results of this study, we predict that outbreaks by *N. parvum* will worsen in warmer summers, possibly thanks to the direct effect of temperature on the growth of the pathogen. However, we note that the magnitude of the increase in in vitro growth rates with an increase of 12 °C in temperature was 1.65-fold (Figure 5). That increase value is much lower than the 3.5- and 7.6-fold increases in the internal and external *in planta* lesion sizes, respectively, which were observed with a similar 12 °C increase in temperature

(Figure 6). We posit that the stronger *in planta* effect may be due to the effects of warmer temperatures on plant physiology or on plant–pathogen interactions, which, in turn, will affect disease severity. In conclusion, our data suggest that temperature affects disease severity through multiple pathways and not solely through affecting fungal growth.

The climatic data from the blueberry production fields currently experiencing the emergence of stem blight and dieback show a prolonged trend of increasing temperatures [60]. In the Piedmont region of Italy, blueberry cultivation consisted of 1461 ha, with 9693 tons of berries produced in 2022, and it is constantly expanding [17]. The results of this study suggest that disease may be curtailed by the selection of growing sites that may be cooler in the summer or may be more resilient to increases in temperature. It should also be considered that in the past decade, blueberry cultivation has expanded into warmer sites with a lower altitude, and blueberry cultural systems have been intensified [61]. Warmer temperatures combined with a high plant density can alter the agro-ecological balance of a plantation as well as the physiology of blueberry plants. The same is true for wounding associated with the mechanization of harvesting given that wounds have been associated with increased infection by fungi in the Botryosphaeriaceae [20,56]. If the changes caused by intensive plantations result in increased wounding and plant stress, we know that infection rates and the virulence of latent pathogens such as *N. parvum* will also increase. Physiological stress will thus trigger disease expression by *N. parvum*, and warmer temperatures, which are ideal for *N. parvum*, will increase the severity of the disease caused by this pathogen [62,63].

We suggest that the productivity of blueberry orchards may be maintained by a mix of strategies that include, but are not limited to, avoiding the introduction of *N. parvum* or similar pathogens, planting in sites selected for a future optimal climate based on climatic predictions, shading the orchard, avoiding excessive wounding and minimizing physiological stress in the host. Assessing the role that each variable above may have on disease may help to further fine-tune disease management strategies. Additional insights may come from studies conducted to investigate the presence of *N. parvum* in asymptomatic plant tissues and commodities other than blueberry and to further understand the biology of this pathogen, which is reported as one of the most widespread causal agents of fungal trunk disease (FTD) due to its broad host range [2,64]. In light of warming trends, generalist pathogens, such as *N. parvum*, truly present a challenge. The enhanced spread and development of this species with rising temperatures could seriously compromise several fruit crops [65]. Thus, further investigations focused on different fruit crops and the genetic structures of both *N. parvum* and host populations should be performed to better our understanding of the ecology and evolution of this pathogen and to improve current management strategies.

Conflicts of Interest: The authors declare no conflicts of interest.

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