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Environmental Requirements for Germination and Appressorium Formation of Ascospores and Conidia of *Phyllosticta citricarpa*, the Causal Agent of Citrus Black Spot

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ABSTRACT

Phyllosticta citricarpa produces ascospores and conidia that infect citrus tissues and cause citrus black spot (CBS). The environmental conditions impact the pre-penetration process of these spores and the CBS intensity worldwide. However, there are few studies related to the optimal conditions for *P. citricarpa* spore germination, especially ascospores. Thus, this study aimed to assess the influence of temperature and wetness duration on germination of both *P. citricarpa* spores. Suspensions of ascospores and conidia produced by *P. citricarpa* were prepared, deposited on polystyrene dishes and kept at temperatures from 10°C to 40°C. Spore germination was assessed from 3 to 48 h of wetness. The beta-monomolecular model was fitted to the data and response surface equations were generated for each spore type. Germination peaks of up to 62% and 90%, and appressorium formation of 61% and 80%, were found for ascospores and conidia, respectively, from 24 h of wetness onwards. Minimum, optimum and maximum temperatures were estimated as 10°C, 24.8°C and 40°C for conidial germination, and 11°C, 24.5°C and 38.4°C for appressorium formation, respectively. The cardinal temperatures estimated for ascospore germination were 9°C, 29.8°C and 39.8°C, and 10°C, 30°C and 39°C for appressorium formation. The differences found between both spores, notably in the optimal temperatures, may influence the role of each type of inoculum in CBS epidemics in the citrus-growing areas worldwide. The response surface models generated in our study are a first step in the development of decision support systems for CBS management, as well as in the disease risk analyses.

1 | Introduction

Citrus black spot (CBS), caused by the fungus *Phyllosticta citricarpa* (syn. *Guignardia citricarpa*), is one of the most severe fungal diseases in citrus orchards, which is widespread in almost all tropical and subtropical citrus-growing regions worldwide (EFSA [European Food Safety Authority] et al. 2020), and in Tunisia, under a Mediterranean climate (Benfradj et al. 2024). CBS is considered an important quarantine disease in several countries, including those in the European Union (EU) (EFSA et al. 2020). The damage caused by this disease includes blemishes to the fruit rind (Kotzé 1981), which lead to quality losses and rejection of fresh fruit exports (Gottwald et al. 2021), as well as premature fruit drop

when severity levels exceed about 4% (Machado et al. 2022). The average CBS-triggered losses caused by fruit drop in the Brazilian citrus belt were estimated to be about $40 million over five seasons from 2016/2017 to 2020/2021 (Moreira et al. 2022).

Phyllosticta citricarpa forms ascospores and conidia. Ascospores are ejected from pseudothecia and spread by the wind (Kotzé 1981; Spósito et al. 2011). They are formed in the presence of isolates from compatible mating types, designated as *MAT1-1* and *MAT1-2*, as *P. citricarpa* is heterothallic (Tran et al. 2017; Wang et al. 2016). The two mating types are present in most citrus-growing countries worldwide, such as Brazil, Australia, South Africa and China (Amorim et al. 2017; Brandão et al. 2024; Coetzeé et al. 2022; Wang et al. 2016), but the pathogen also develops epidemics with clonal populations, which reproduce solely through conidia, as occurs in Florida, Cuba and Tunisia (Ioos et al. 2024; Serra et al. 2022; Wang et al. 2016). Ascospores were produced and ejected at temperatures from 15°C to 25°C in laboratory conditions, while no ascospores were ejected from pseudothecia at 10°C and 30°C (Brandão et al. 2024). In the orchard, ascospores of mixed *Phyllosticta* spp. populations were produced in leaf litter under drying and wetting conditions (Kotzé 1981), and the ascospore release was observed from 16°C to 32°C, with 22°C as the average temperature (Moyo et al. 2020). Conidia are asexually produced in pycnidia formed in leaf litter, diseased fruit with hard, freckle and virulent spots, and dead twigs (Kotzé 1981; Tran et al. 2020). Conidia are mainly spread by rain splash to short distances in the citrus canopies (Kotzé 1981; Spósito et al. 2011), although this distance may increase with rains driven by the wind (Hendricks et al. 2017, 2020; Perryman et al. 2014).

Temperature and wetness duration are the most studied environmental requirements affecting spore germination of fungal pathogens (Bonde et al. 2007; Guzman-Plazola et al. 2003; Lima et al. 2011; Rasera et al. 2022). The effects of these factors on germination of *P. citricarpa* spores were assessed only for conidia in three studies (Korf 1998; Noronha 2002; Wang and Dewdney 2019). Noronha (2002) performed assays with an isolate at seven temperatures (from 10°C to 40°C) and four wetness durations (from 12 to 48 h), and expressed the appressorium formation as relative percentage and a beta-monomolecular model was proposed. Korf (1998) assessed the conidial germination of four *P. citricarpa* isolates from different countries at seven temperatures (from 0.5°C to 40°C) after 48 h of wetting; however, molecular *Phyllosticta* species identification was not performed, and endophytic species may have been used due to their morphological similarity with *P. citricarpa* (Baayen et al. 2002). Wang and Dewdney (2019) assessed the conidial germination and appressorium formation of an isolate at nine temperatures (from 4°C to 32°C) for 24 h and at 24°C in 10 wetness durations (from 2 to 36 h), but they did not generate a surface response model combining these two environmental variables. There is no work assessing the effect of temperature and wetness on the germination and appressorium formation of *P. citricarpa* ascospores, probably due to a protocol for *P. citricarpa* ascospore production in the laboratory only recently being developed (Tran et al. 2017) and the difficulty of producing them in large quantities (Brandão et al. 2024). In addition, trials performed in the citrus orchards with conventional spore traps (Fourie et al. 2013; Moyo et al. 2020; Reis et al. 2006) probably generated data for *P. citricarpa* and *P. capitalensis*, as both species produce morphologically similar ascospores (Baayen et al. 2002). Thus, the difficulty of separating *Phyllosticta* spp. ascospores under field conditions reinforces the need to perform experiments with this inoculum produced under laboratory conditions.

The effects of environmental variables on germination of conidia and ascospores of ascomycetous plant pathogens have been variable. Some studies performed with *Botryosphaeria obtusa*, the causal agent of fruit black rot in apple, and *Didymella rabiei* that affects chickpea, showed that conditions required for germination of conidia and ascospores were similar (Arauz and Sutton 1989; Trapero-Casas and Kaiser 2007). On the other hand, a work with *Neonectria ditissima* in apple reported that the optimal germination temperature for ascospores ranged from 18°C to 21°C, lower than those estimated for conidia, which ranged from 21°C to 24°C (Gelain et al. 2024). In the case of *Phyllosticta* species, there are other factors affecting conidial germination, such as the surface where the spores are deposited (e.g., hydrophobic or hydrophilic) and physicochemical conditions of the spore suspension (Shaw et al. 2006; Wang and Dewdney 2019). Conidia of *P. citricarpa* did not germinate in sterilised water even on a hydrophobic surface at 24°C (Wang and Dewdney 2019), demonstrating that nutritional conditions are required for triggering *P. citricarpa* conidial germination. It is likely that *P. citricarpa* ascospores have similar requirements, but studies need to be performed with this inoculum. In addition, the assessment of appressorium formation is important not only because the conditions required for its formation may differ from those necessary for germ tube formation, but also this specialised structure is used by many pathogens to infect hosts (Chumley and Valent 1990; Steiner and Oerke 2007). Appressoria may also endure adverse environmental conditions such as UV light and desiccation, increasing the chance of successful infection (Emmett and Parbery 1975).

An understanding of the environmental requirements for the ascospore germination process is important as this inoculum may introduce CBS into new areas, because the pathogen may be harboured asymptomatically in infected materials (Gottwald et al. 2021), and when the leaves drop in a new location with favourable climate conditions, these spores are produced and ejected (Kotzé 1981).

Moreover, modelling the relationship among temperature, wetness duration and germination or appressorium formation of both *P. citricarpa* spores may serve as a basis for analysing risks of disease establishment in CBS-free areas worldwide (Galvañ et al. 2022; Yonow et al. 2013), as well as for developing a CBS predictive system, after proper validation under field conditions, to better manage this disease.

Based on the lack of studies on environmental conditions affecting the pre-penetration process of *P. citricarpa* ascospores, the aims of this study were to (i) assess the effect of different temperatures and wetness durations on *P. citricarpa* ascospore and conidial germination; (ii) compare the environmental

requirements for the pre-penetration process among isolates collected from the São Paulo citrus belt; and (iii) develop response surface models with the relationship among temperatures, wetness durations and germination/appressorium formation for conidia and ascospores of *P. citricarpa*.

2 | Materials and Methods

2.1 | Selection and Identification of *P. citricarpa* Isolates

A total of eight single-spore *P. citricarpa* isolates were used in this study. The collection and species and mating type identification were performed by Brandão et al. (2024). These isolates were selected due to their mating-type compatibility and diverse regional origins from the São Paulo citrus belt, in which they were collected. The isolates 1 (LRS 42/12, *MAT1-1*) and 5 (FDC 04/16, *MAT1-2*) were sampled in the Center region, isolates 2 (FDC 46/21, *MAT1-2*) and 6 (FDC 39/21, *MAT1-1*) were from the North region and isolates 3 (FDC 09/17, *MAT1-2*), 4 (FDC 17/09, *MAT1-2*), 7 (LRS 18/13, *MAT1-1*) and 8 (FDC 12/09, *MAT1-1*) were from the South region. The regions were selected due to differences in their climate classification: Center—mainly Cfa (humid subtropical zone with hot summers and no dry season); South—Cfa or Cwa (humid subtropical zone with dry winter and hot summer); and North—mainly Aw (tropical zone with dry winter), according to Koppen's climate classification (Alvares et al. 2013). These isolates were stored at 7°C using the sterilised filter paper method (Silva Junior et al. 2016) and subcultured to dishes containing 15 mL of potato dextrose agar (PDA; Difco) to use in the following steps.

2.2 | Ascospore and Conidia Production

The ascospores were produced using a spermatia suspension as described by Tran et al. (2017) and modified by Brandão et al. (2024). Colonies of the eight *P. citricarpa* isolates were grown on 15 mL of PDA at 25°C and 12 h of light for 2 weeks. Four crosses were used: Cross 1—isolate 1 × isolate 5 (Center); Cross 2—isolate 2 × isolate 6 (North); Cross 3—isolate 3 × isolate 7 (South); Cross 4—isolate 4 × isolate 8 (South). The dishes containing the crosses were stored for 21 days at 25°C and 12 h of light. Next, the dish lids were left for 14 days after the crosses and then removed and replaced by sterile lids to collect the ascospores ejected from 14 to 21 days after the crosses, given that the viability of *P. citricarpa* ascospores may decrease over time after their ejection (Beyer and Verreet 2005). The ejected ascospores were collected by adding 1 mL of a 2% sterilised sweet orange (*Citrus sinensis* 'Valencia') juice solution on the dish lids and scraping their surfaces with a Drigalski spatula. This solution was prepared by diluting 2% squeezed juice with sterile distilled water containing Tween 20 at 0.02%, then homogenising it in a vortex mixer. The resulting solution was sterilised with a 0.2-μm polyethersulfone (PES) membrane filter. The characteristics of the juice were °Brix = 12.49; titratable acidity (%) = 1.07; Ratio (soluble solids/titratable acidity) = 11.68; pH = 3.65; vitamin C = 492 mg/L. The collected ascospore suspensions were transferred to Falcon tubes of 50 mL, homogenised in a vortex mixer, and the ascospore concentration adjusted from 10^3–10^4 ascospores/mL in a Neubauer chamber, depending on the ascospore production (Brandão et al. 2024).

The conidial suspensions were prepared according to the method of Wang and Dewdney (2019), with some modifications. Colonies of four *P. citricarpa* isolates (isolates 1, 2, 3 and 4) were grown on 15 mL of PDA for 2 weeks at 25°C and 12 h light. The conidial suspensions were prepared by removing two mycelial disks of approximately 4.5 mm from colonies and placing them into Falcon tubes containing 50 mL of the 2% sterilised sweet orange juice solution described above, homogenising the suspensions in a vortex mixer, and then filtering the spore suspension through a sterile cheesecloth. The concentration was adjusted to 10^4 conidia/mL in a Neubauer chamber.

2.3 | Comparison of the Ascospore and Conidium Germination and Appressorium Formation Among *P. citricarpa* Isolates

The experiment aiming to verify whether germination and appressorium formation vary with the origin of the isolates was performed in a completely randomised design, with four *P. citricarpa* isolates (1 to 4) to produce conidia and four crosses (1 to 4) to produce ascospores, at three temperatures (15°C, 25°C and 35°C) and three wetness durations (12, 24 and 48 h). Three drops of 50 μL of the spore suspension were deposited equidistantly on polystyrene Petri dish lids. Wetted cotton fragments were placed in the lids and dishes sealed with Parafilm to avoid drop evaporation. Each dish with the drops was placed into a Gerbox containing filter paper wetted with 10 mL water to keep the moisture high within the dish. The dishes were transferred to growth chambers configured with each temperature and a light period of 12 h. Temperature (°C) and relative humidity (%) were recorded hourly over time with thermohygrometers (model RHT-LCD; Novus). These recorded temperatures were used to estimate the average temperature during each wetness duration, which was used in the statistical analyses.

Ascospore and conidium germination and appressorium formation were assessed after the respective hours of wetness by removing the dishes from the incubators, adding 40 μL of lactoglycerol to the spore suspension drops to stop germination, and storing them at 5°C (Korf 1998; Noronha 2002) until the assessments. The assessments were performed with light microscopy at a 400× magnification. When the germ tube length was equal to or longer than the spore length or when the melanised appressorium was observed, the spore was considered germinated. A total of 100 ascospores or conidia were assessed per drop. The possible effect of high temperatures on spore form was assessed by measuring the length of 10 spores kept at a given temperature compared to 10 spores at 25°C. The experiment was conducted with two factors (3 × 3), the first one consisting of the three temperatures and the second one the three wetness durations. The factor isolates or crosses was analysed separately to check if there was a difference among them and thereafter they were analysed pooled or separately. Four replicates per treatment were used, and each one consisted of three drops of the spore suspension on a dish lid. The experiment was undertaken twice.

2.4 | Ascospore and Conidium Germination and Appressorium Formation Under Diverse Temperatures and Wetness Durations

The experiment to generate the response surface curve for ascospore and conidium germination and appressorium formation as a function of temperature and wetness durations was performed with isolate 1 to produce conidia and cross 1 to produce ascospores. This isolate and cross were selected because the central region of São Paulo citrus belt has a relevant production area generally affected by CBS (Fundecitrus 2023). The experiment was conducted as a completely randomised design, with two factors (7 × 5) consisting of seven temperatures (10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C) and five wetness durations (3, 12, 24, 36 and 48 h). The methodology and assessment were the same as described before for the experiment performed to compare isolates and crosses. The experiment was conducted with four replicates per treatment and undertaken twice.

2.5 | Statistical Analysis

The data of each experiment and its replicate were pooled when homogeneity of variances was detected using the Hartley's *F*-ratio test at 5% significance (Hartley 1950; Snedecor and Cochran 1989). Comparison of the germination and appressorium formation of the four crosses and isolates at the three temperatures and wetness durations was performed using a generalised linear model (GLM) nonparametric approach and Gaussian distribution, given that these data did not fulfil ANOVA assumptions, even after Box–Cox transformation. The means were compared with the post hoc Tukey's test at 5% significance.

Nonlinear regression analysis was used to generate the response surface curve equations of germination/appressorium formation for ascospores and conidia as a function of temperature (from 10°C to 40°C) and wetness durations (from 3 to 48 h). For this, the germination and appressorium formation data of ascospores and conidia were transformed into proportion (Madden et al. 2007) to fit to the beta-monomolecular model, obtained by the multiplication of the beta generalised (Bassanezi et al. 1998) and monomolecular models (Bergamim Filho et al. 2018). Thus, the response surface equation obtained was

$$Z(T,WP)=Y_{opt}\times(((T-T_{min})/(T_{opt}-T_{min}))\wedge(b_1\times(T_{opt}-T_{min})/(T_{max}-T_{opt})))\times(((T_{max}-T)/(T_{max}-T_{opt}))\wedge(b_1)))\times(Y_{opt}-(Y_{opt}-y_0)\times\exp(-r\times WP))$$

in which Z (T, WP) is the germination or appressorium formation in proportion at a specific temperature (T) and wetness duration (WP); Y_{opt} is the proportion of spores germinated or with appressorium at the optimal temperature; T_{min}, T_{opt} and T_{max} are the minimum, optimum and maximum temperatures, respectively; b_1 is the range of temperatures for germination or appressorium formation, that is, the higher this parameter is, the narrower the range of temperatures is; y_0 is the germination or appressorium formation at zero hours of wetness, and r is the germination or appressorium formation rate. The quality of data fit to this model was assessed by the coefficient of determination (R^2), obtained by a linear regression analysis between the estimated and observed data, observation of the random distribution and values near zero of the residuals, and values of the root mean square error (RMSE) (Campbell and Madden 1990). The estimated parameters of ascospore and conidium germination and appressorium formation were compared by the Student's *t* test at 5% significance. This test was also used to check if the parameters were different from zero and to compare the average length of spores with visual difference in form due to high temperatures. The nonlinear regression analyses were performed in XLSTAT (Addinsoft) and the other analyses and figures in the SigmaPlot v. 12.0 software (Systat Software Inc.). The GLM analyses were performed using the software R v. 3.6.1 (R Core Team 2019).

3 | Results

3.1 | Comparison of the Ascospore and Conidium Germination and Appressorium Formation Among *P. citricarpa* Isolates

The ascospore germination of the four crosses occurred at the three temperatures and wetness durations assessed (Figure S1), whose minimum and maximum average values varied from 5% to 40%, respectively (Figure 1a–d). There was difference among the crosses for germination (Figure 2a), which were analysed separately. Interaction among both factors was detected in crosses 2 and 3 ($p < 0.05$). In most of the wetness durations, the germination was higher at 25°C, followed by 35°C, and the lowest values observed at 15°C (Figure 3a–d). The average peaks of 30%, 32%, 38% and 17% were observed for crosses 1, 2, 3 and 4, respectively. The germination peaks occurred mostly from 24 h of wetness onwards, with the lowest average values of 10%, 13%, 14% and 7% for crosses 1, 2, 3 and 4, respectively, observed at 12 h (Figures 1a–d and 3a–d). The ascospore appressorium formation occurred in all temperatures and wetness durations, with minimum and maximum average values varying from 2% to 37%, respectively (Figure 1e–h). There was difference among the crosses for appressorium formation (Figure 2b), which were analysed separately. Interaction among both factors was detected in cross 3 ($p < 0.05$). In most crosses, the appressorium formation was higher at 25°C and 35°C, and the lowest values observed at 15°C (Figure 3e–h). The appressorium formation average peaks of 17%, 18%, 31% and 10% were observed for crosses 1, 2, 3 and 4, respectively (Figure 1e–h). In some crosses and temperatures, the appressorium formation peaks occurred mostly from 24 h of wetness onwards, with the lowest average values of 10%, 13%, 14% and 7% for crosses 1, 2, 3 and 4, respectively, observed at 12 h (Figures 1e–h and 3e–h).

The conidial germination of the four isolates occurred in the three temperatures and wetness durations assessed, whose minimum and maximum average values varied from 40% to 90%, respectively (Figure 1i–l). There was difference among the isolates for germination (Figure 2c), which were analysed separately. Interaction among both factors was detected in the four isolates ($p < 0.05$). In most of the wetness durations,

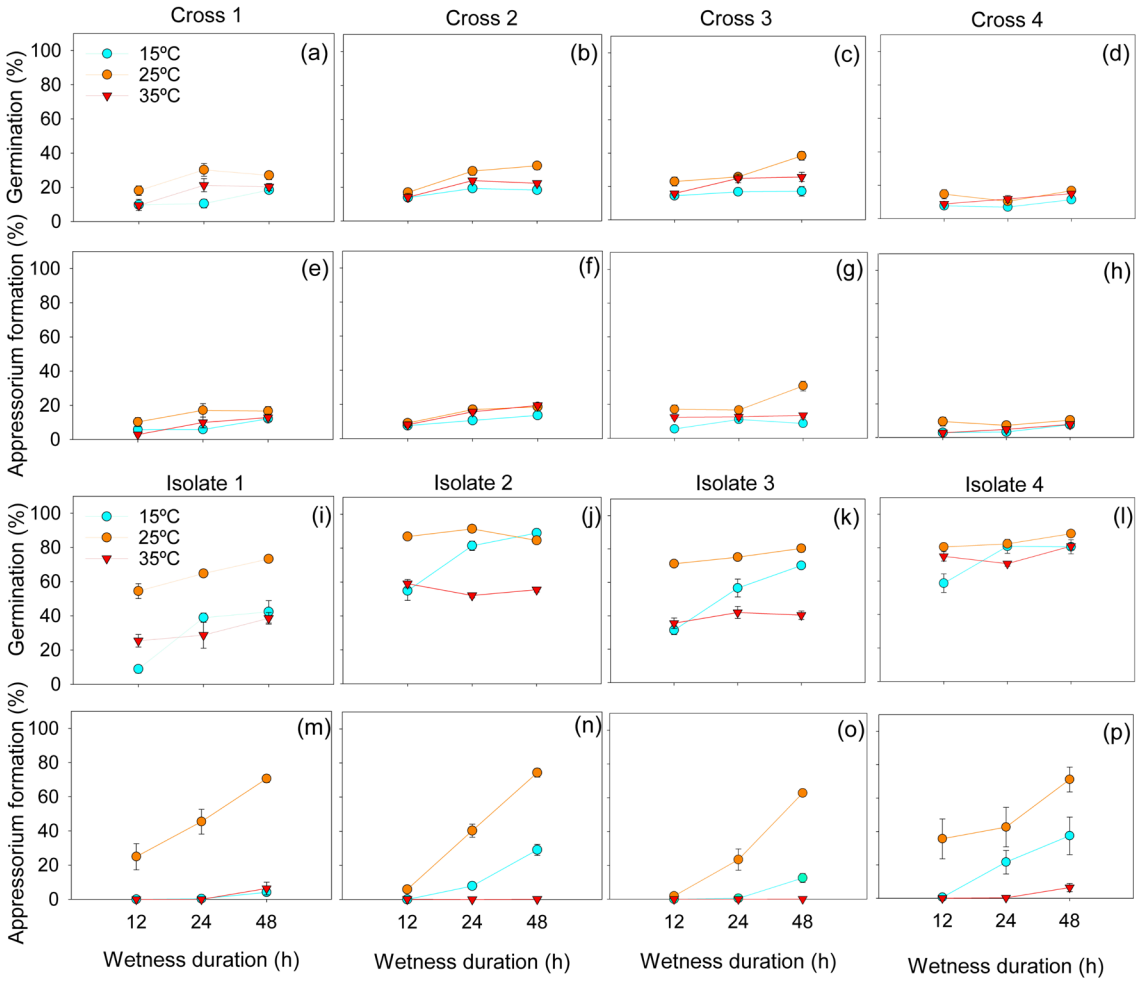

FIGURE 1 | Percentage of ascospore germination (a–d) and appressorium formation (e–h), and conidial germination (i–l) and appressorium formation (m–p) from four *Phyllosticta citricarpa* crosses and isolates obtained in different regions of the São Paulo citrus belt at 15°C, 25°C and 35°C after 12, 24 and 48 h of wetting in suspensions containing 2% of sweet orange juice over polystyrene dish lids. Dots represent the average germination and the error bars the standard error (pooled data from both experiments). [Colour figure can be viewed at wileyonlinelibrary.com]

the germination was higher at 25°C, followed by 15°C, and the lowest values observed at 35°C, except for the isolates 1 and 4 (Figure 3i–l). Conidial germination average peaks of 73%, 91%, 80% and 88% were observed for isolates 1, 2, 3 and 4, respectively. The germination peaks occurred mostly from 12 h of wetness onwards at 15°C. The lowest average values of 10%, 54%, 31% and 58% for isolates 1, 2, 3 and 4, respectively, were observed at 12 h (Figures 1i–l and 3i–l). At 35°C and after 12 h of wetting onwards, the conidia were swollen and bigger (average length = 12.14 ± 0.50 μm) in comparison to the normal ones (9.88 ± 0.37 μm), with atypical germ tubes (Figure S2) and significant difference in their average length ($p < 0.05$). The appressorium formation was higher at 25°C, followed by 15°C and almost no appressoria were observed at 35°C (Figure 1m–p) in most wetness durations and isolates (Figure 3m–p). The appressorium formation average peaks of 70%, 72%, 62% and 71% were observed for the isolates 1, 2, 3 and 4, respectively (Figure 1m–p). The appressorium formation peaks for all isolates occurred mostly between 24 and 48 h of wetness, with the lowest average values of about 0% observed at 12 h at 15°C and 35°C (Figures 1m–p and 3m–p).

3.2 | Ascospore and Conidial Germination and Appressorium Formation Under Diverse Temperatures and Wetness Durations

In both experiments performed with ascospores produced by cross 1, germination peaks of up to 62% (average of ~42%) occurred at 30°C after 24 h of wetness onwards. High levels of ascospore germination (up to 42%) were also observed at 25°C after 24 h of wetness. Germination rates from 10% to 20% were observed for ascospores kept at 15°C, 20°C or 35°C for at least 24 h of wetness. The lowest germination percentages of ~2% were observed at 10°C and 40°C in less than 12 h of wetness (Figure 4a). Germination rates from 0% to 7% were observed at 3 h, 10% to 40% after 12 h, and some replicates showed a peak of approximately 62% from 24 h of wetness onwards (Figure 4a). Appressorium formation of ascospores occurred from 15°C to 35°C (Figure S1), with peaks of up to ~30% at 25°C and ~60% (average of 41%) at 30°C from 24 h of wetness onwards. Rates of appressorium formation ranged from 1% to 20% at temperatures of 15°C and 20°C, and from 0% to 3% at 35°C in wetness durations longer than 24 h. Almost no appressoria were

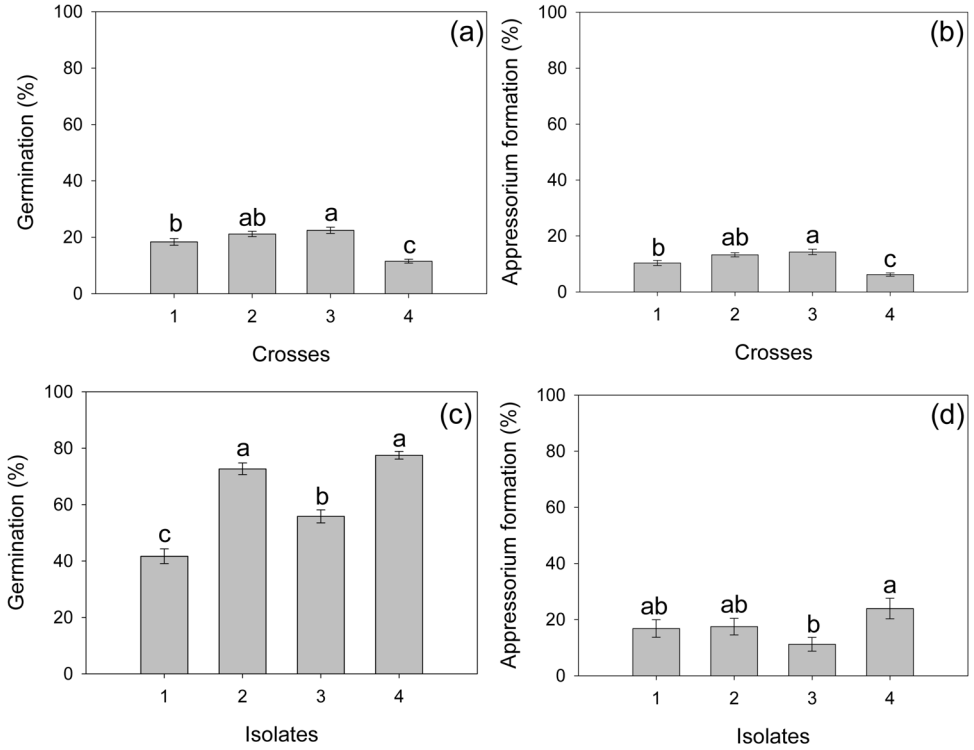

FIGURE 2 | Ascospore (a, b) and conidial (c, d) germination (a, c) and appressorium formation (b, d) of four *Phyllosticta citricarpa* crosses and isolates from four regions of the São Paulo citrus belt in suspensions containing 2% of sweet orange juice over polystyrene dish lids. Means followed by the same letters do not differ from each other by the Tukey's test at 5% significance. Pooled data from both experiments. Error bars represent the standard error.

observed at 10°C and 40°C (Figure S1), irrespective of the wetness, and at all temperatures tested in the shortest wetness duration of 3 h (Figure 4b). No swollen ascospores, that is, bigger ascospores than regular ones, were observed at the maximum and minimum temperatures assessed (Figure S1). The beta-monomolecular model was significant ($p < 0.05$) when adjusted to the data of ascospore germination (RMSE = 0.089) and appressorium formation (RMSE = 0.084), with R^2 values ≥ 0.60 (Table 1) (Figure 4c,d). This means at least 60% of variance in ascospore germination and appressorium formation is explained by both independent variables (temperature and wetness duration) with the equation obtained. The comparison of estimated parameters between germination and appressorium formation showed no differences among all the estimated parameters ($p > 0.05$) (Table 1).

The conidial germination increased as the wetness duration increased in both experiments conducted with isolate 1 (Figure 5a and Figure S2). Germination peaks of up to 80% (average of ~78%) were observed when the conidia were kept at 25°C from 24 h of wetness onwards. High germination rates of 81% and 66% were also observed at 20°C and 30°C, respectively, while rates from 0% to 55% were found at 15°C and from 0% to 38% at 35°C (Figure 5a). Fewer than 20% of conidia germinated at 10°C and rarely at 40°C (Figure 5a and Figure S2). The lowest germination rates from 0% to 2.3% were observed within 3 h of wetness. Periods of 12 h of wetness were enough for the germination of up to 52% of conidia, whereas the maximum rates occurred from 12 to 24 h of wetness onwards depending on the assessed temperatures (Figure 5a). Appressorium formation reached peaks of 75% at 20°C and 25°C mostly after 24 h of wetness onwards (Figure 5b). Rates of up to 15% were observed at 15°C, and values lower than 10% at 35°C in the two experiments. Almost no appressoria were observed at 10°C and 40°C or after 3 h of wetness in all temperatures (Figure 5b), and swollen conidia were observed from 35°C onwards (Figure S2). The beta-monomolecular model also showed a good fit to data of conidial germination (RMSE = 0.095) and appressorium formation (RMSE = 0.087), with R^2 values ≥ 0.80 (Table 1 and Figure 5c,d). This means at least 80% of variance in the conidial germination and appressorium formation is explained by both independent variables (temperature and wetness duration) with the equation obtained. The Y_{opt}, T_{max} and r parameters were significantly higher, while b_1 was lower for germination than appressorium formation ($p < 0.05$) (Table 1).

The comparison of the estimated parameters for conidium and ascospore germination showed that T_{opt} was higher for ascospores (29.81) compared to conidia (24.80), whereas Y_{opt} was higher for conidia (0.86) than ascospores (0.65). The values estimated for the other parameters (T_{min}, T_{max}, b_1, y_0 and r) were not significantly different for ascospores and conidia (Table 1). In the case of appressorium formation, T_{opt} was higher for ascospores (30.03) than conidia (24.47), while Y_{opt} and b_1 were higher for conidia (0.72 and 3.00) compared to ascospores (0.63 and 2.00) (Table 1). All the values estimated for each parameter were different from zero, except for the y_0 parameter estimate to zero for both pre-penetration processes of ascospores and conidia (Table 1).

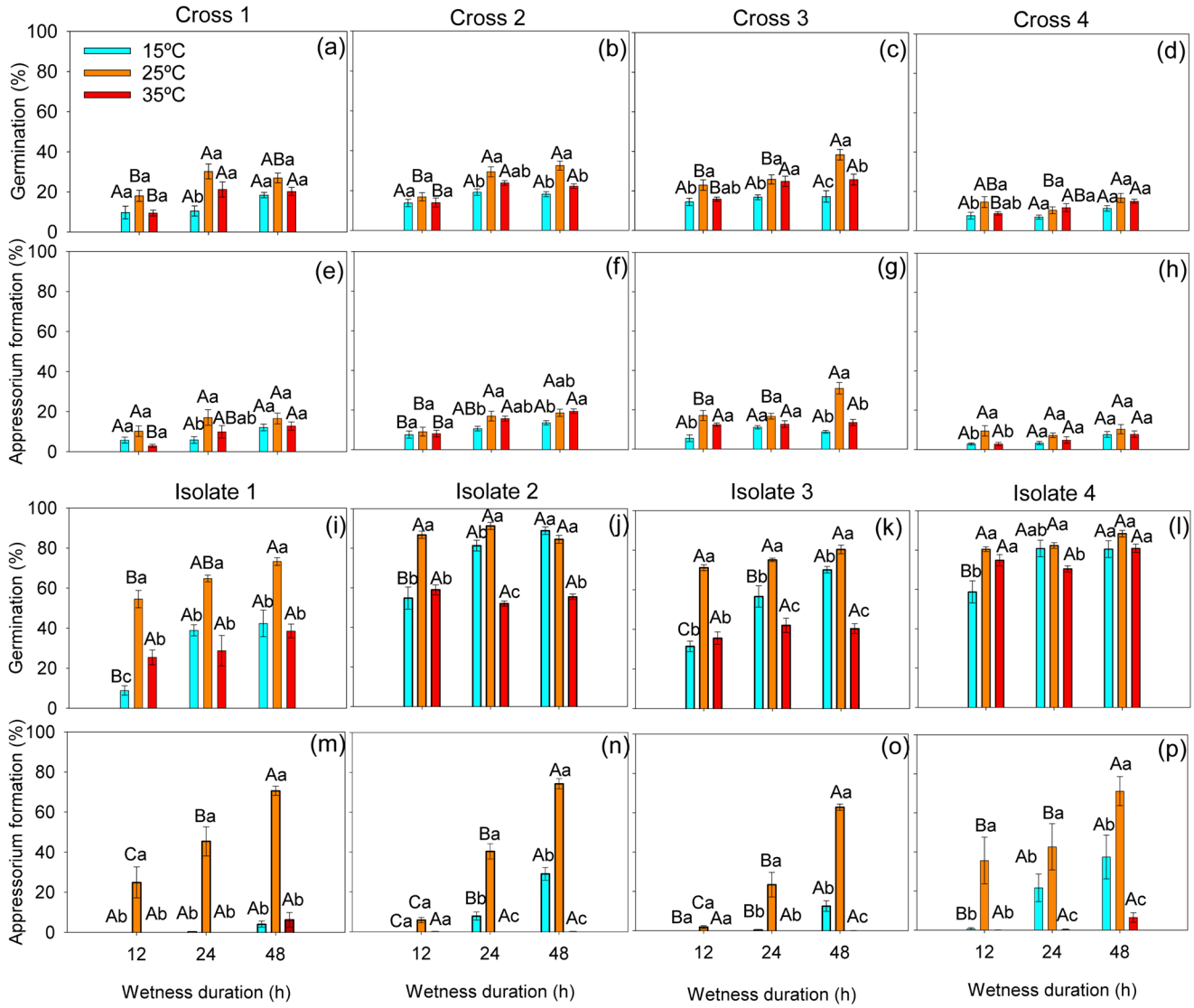

FIGURE 3 | Percentage of ascospore germination (a–d) and appressorium formation (e–h), and conidial germination (i–l) and appressorium formation (m–p) from four *Phyllosticta citricarpa* crosses and isolates obtained in different regions of the São Paulo citrus belt at 15°C, 25°C and 35°C after 12, 24 and 48 h of wetting in suspensions containing 2% of sweet orange juice over polystyrene dish lids. Means followed by the same uppercase letter, comparing one temperature in the three wetness durations, and lowercase letter, comparing the three temperatures in one wetness duration, do not differ from each other by the Tukey's test at 5% significance. Error bars represent the standard error (pooled data from both experiments). [Colour figure can be viewed at wileyonlinelibrary.com]

4 | Discussion

This study characterised for the first time the combined effect of temperature and wetness duration over the pre-penetration processes of *P. citricarpa* ascospores and conidia. The beta-monomolecular model estimated that the optimal temperatures for ascospore germination and appressorium formation were higher than those for conidia. Furthermore, conidia had greater germination and appressorium formation when compared to ascospores, regardless of the temperature, wetness duration and isolates assessed.

In general, ascospores and conidia from all four isolates and crosses tested in our study collected from the South to the North of the São Paulo citrus belt had similar germination rates when tested in low, median and high temperatures; however, some isolates and crosses showed differences from each other. The similarity observed among some isolates was probably because the average temperatures in the North São Paulo citrus belt, mostly covered by Aw climate, are on average only 1°C–2°C higher compared to the South regions that have Cfa climate (Alvares et al. 2013). Our results corroborate Korf (1998), who observed that the highest conidial germination and appressorium formation of four *Phyllosticta* isolates from Brazil, India and South Africa occurred at 22°C. Er et al. (2014) estimated the optimal temperature to be ~26°C for mycelial growth of three *P. citricarpa* isolates, and Brandão et al. (2024) also found no difference in ascospore production among *P. citricarpa* crosses at five different temperatures. Regarding other ascomycetous fungi, Úrbez-Torres et al. (2010) also did not observe significant differences for conidial germination among most isolates of species belonging

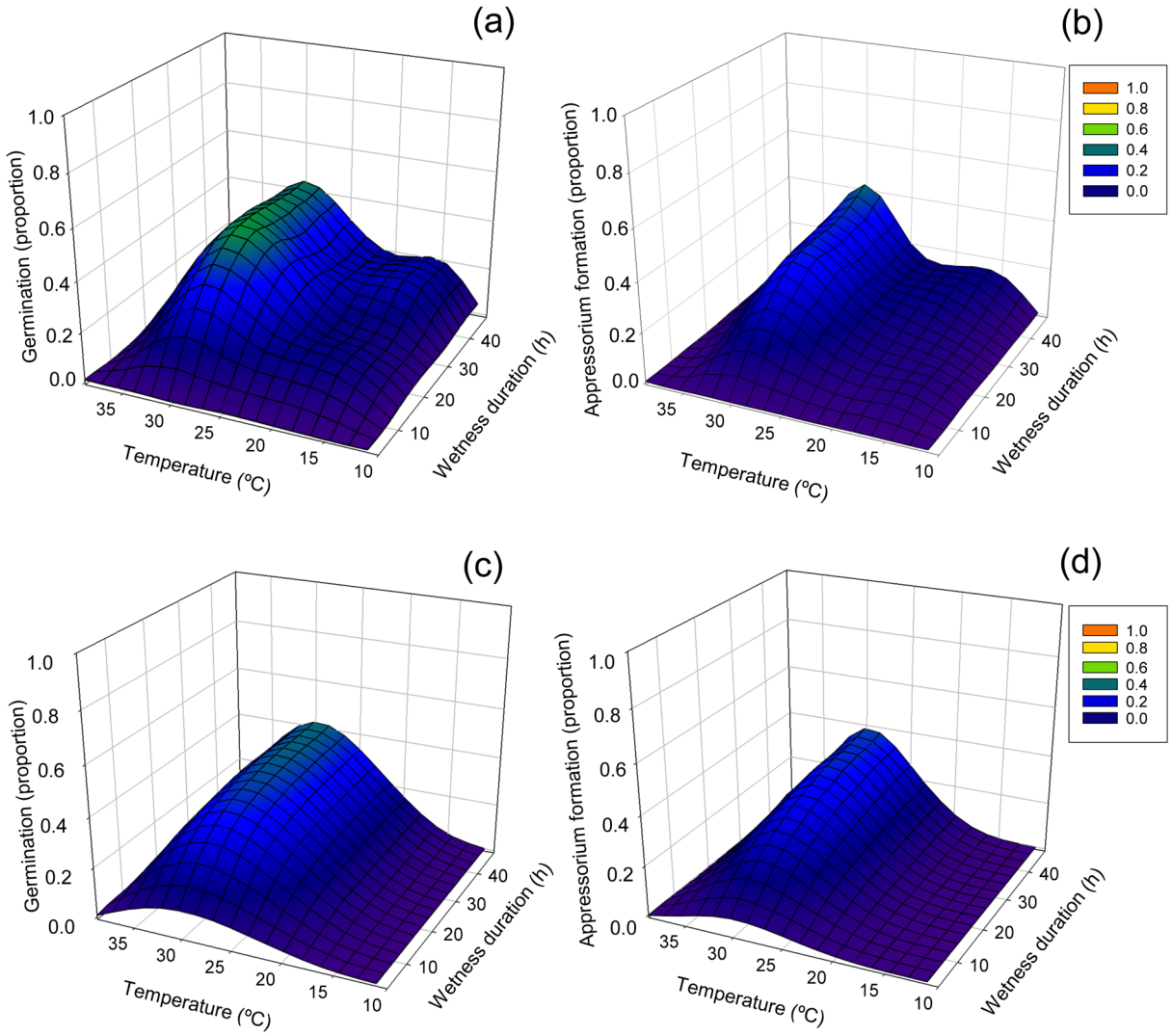

FIGURE 4 | Comparison of response surface curves with observed (a,b) and estimated (c,d) data using the beta-monomolecular model obtained from the average *Phyllosticta citricarpa* ascospore in vitro germination (a, c), and appressorium formation (b, d) in proportion (*z*-axis) under diverse temperatures (*x*-axis) and wetness durations (*y*-axis). Pooled data from both experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

to the *Botryosphaeriaceae* family sampled from grapevines and kept under different temperatures; however, these authors used only two isolates from some *Botryosphaeriaceae* species assessed. It is important to mention that variations observed in our study were mostly between the two experiment repetitions rather than among isolates or crosses. Temperature fluctuations in the growth chambers were also a source of variation, because in the experiments with conidia a larger standard error was observed, for example, at 15°C than at 25°C. Thus, further studies with more isolates from other citrus-growing regions are necessary to assess if *P. citricarpa* isolates from some regions require similar environmental conditions for ascospore and conidial germination.

The estimated cardinal temperatures of about 10°C, 30°C and 40°C were similar for the germ tube and appressoria formation processes of *P. citricarpa* ascospores. On the other hand, conidia formed the germ tube and appressoria under the similar minimum (~10°C) and optimal (~24.5°C) temperatures, but a significant difference (40°C vs. 38.4°C) was observed for the maximum temperatures in which these processes occur. Wang and Dewdney (2019) and Korf (1998) found ~10°C–12°C, ~22°C–24°C and ~35°C–36°C as minimum, optimum and maximum temperatures, respectively, for *P. citricarpa* conidial germination, while Noronha (2002) estimated the minimum and maximum temperatures for appressorium formation from conidia at 3°C and 48°C, respectively. These differences among the estimated cardinal temperatures in the studies with *P. citricarpa* conidia may be associated with the surface and nutritional conditions that affected the pre-penetration process (Shaw et al. 2006; Wang and Dewdney 2019). In our study, the surface used to assess the germination was polystyrene, whereas Wang and Dewdney (2019) used polytetrafluoroethylene (PTFE) (Teflon) as the hydrophobic surface. Despite PTFE being more hydrophobic than polystyrene, both surfaces allowed similar conidial germination percentages in a previous study with other *Phyllosticta* species (Kuo and Hoch 1996). Thus, this influence of the surface on the pre-penetration processes suggests the need to perform studies on citrus leaves or fruit peel, simulating the natural conditions in which *P. citricarpa* infections occur.

TABLE 1 | Estimated parameters (± standard error) and adjusted determination coefficient (R^2) estimated by the beta-monomolecular model fitted to the germination and appressorium formation data of *Phyllosticta citricarpa* ascospores and conidia under different temperatures and wetness durations.

	Estimated parameter							
Variable	Y_{opt}	T_{min}	T_{opt}	T_{max}	b_1	y_0	r	R^2
Germination								
Ascospores	0.65 Ab ± 0.00	9.00 Aa ± 4.47	29.81 Aa ± 0.36	39.75 Aa ± 0.64	1.47 Aa ± 0.36	0.00 Aa ± 0.06	0.09 Aa ± 0.01	0.66
Conidia	0.86 Aa ± 0.01	10.00 Aa ± 0.00	24.80 Ab ± 0.24	40.00 Aa ± 0.00	1.18 Ba ± 0.07	0.00 Aa ± 0.05	0.10 Aa ± 0.01	0.86
Appressorium formation								
Ascospores	0.63 Ab ± 0.03	10.00 Aa ± 6.80	30.03 Aa ± 0.49	39.03 Aa ± 0.60	2.00 Ab ± 0.00	0.00 Aa ± 0.06	0.05 Aa ± 0.01	0.60
Conidia	0.72 Ba ± 0.03	11.00 Aa ± 1.87	24.47 Ab ± 0.35	38.39 Ba ± 0.77	3.00 Aa ± 0.00	0.00 Aa ± 0.05	0.05 Ba ± 0.01	0.80

Note: beta-monomolecular model: $Z(T, WP) = Y_{opt} \times \{[(T - T_{min}) / (T_{opt} - T_{min})]^{[b_1 \times (T_{opt} - T_{min}) / (T_{max} - T_{opt})]}\} \times \{[(T_{max} - T) / (T_{max} - T_{opt})]^{b_1}\} \times [Y_{opt} - (Y_{opt} - y_0) \times \exp.(-r \times WP)]$, in which Z is the germination or appressorium formation given in proportion; T is the temperature; WP is the wetness duration; Y_{opt} is the germination at the optimum temperature; T_{min}, T_{opt} and T_{max} are the minimum, optimum and maximum estimated temperatures, respectively; b_1 is the range of temperatures for germination or appressorium formation; y_0 is the germination or appressorium formation at zero hours of wetness; and r is the germination or appressorium formation rate. Uppercase letters were used to compare the parameters between germination and appressorium formation separately for each spore type and lowercase letters were used to compare the parameters between ascospores and conidia separately for each prepenetration process by the Student's t test at 5% significance ($p < 0.05$).

Although the minimum (~10°C) and maximum (~40°C) temperatures for the pre-penetration process of *P. citricarpa* ascospores and conidia were similar, the optimal temperature for ascospore germ tube and appressoria formation was estimated by the model to be ~5°C higher than that for conidia. In addition, ascospores formed appressoria and did not become swollen at temperatures greater than 35°C as occurred for conidia. These results suggest that the peaks of citrus infections by each *P. citricarpa* spore may occur under different weather conditions in the orchards, and the ascospores seem to be more adapted to warmer seasons than conidia. As observed in our study, differences in temperatures required by both sexual and asexual spores to germinate were also observed in other diseases caused by ascomycetes in a Mediterranean climate, for example *N. ditissima*, the causal agent of European apple canker, which has an optimal temperature from 18°C to 21°C for ascospore germination, lower than 21°C to 24°C estimated for conidia (Gelain et al. 2024). In California, ascospores of this pathogen are responsible for primary infections, and they are mostly captured by spore-traps from winter to cool spring (November to March) (Dubin and English 1974) when temperatures are lower and rainfall is higher, whereas conidia are generally present whenever the temperature is above freezing conditions and there is sufficient moisture (Weber 2014). Ascospores of *Venturia inaequalis*, the causal agent of apple scab, germinated at minimum, optimum and maximum temperatures of 0.5°C, 16°C–20°C and 30°C, while these temperatures for conidia were 2°C, 16°C–25°C and 32°C, respectively (Boric 1985). Most ascospores of *V. inaequalis* are captured in the winter at average temperatures between 8.4°C and 20.3°C, although ejection may occur from 1°C, albeit very slowly, to over 20°C (Rossi et al. 2003), whereas conidia are rarely detected on the exterior surfaces of overwintered buds or lesions on infected shoots (Becker 1992). On the other hand, the causal agent of Ascochyta blight in chickpea, *D. rabiei*, showed no difference between the optimal temperatures for ascospore and conidial germination (~20°C) (Trapero-Casas and Kaiser 2007), and more ascospores were also captured from winter to cool spring season in Spain, with infections by this inoculum occurring mostly in early spring (Trapero-Casa et al. 1996).

In the case of CBS, which is a disease typical of tropical and subtropical climates (EFSA et al. 2020; Yonow et al. 2013), that is, with rainy summers and dry winters, we hypothesise that the higher optimal temperature required for ascospore germination may be due to rainy season occurrence in summer, in which ascospores are produced concomitantly with conidia (Fialho et al. 2024; Fourie et al. 2013; Reis et al. 2006; Tran et al. 2020). Consequently, *P. citricarpa* ascospores may cause secondary infections along with conidia, but when the temperature starts to decrease conidial infections probably become more important. This concomitant production of both asexual and sexual spores also occurs with other fungal diseases caused by ascomycetes in tropical and subtropical climates, such as banana yellow sigatoka, caused by *Mycosphaerella musicola* (Rocha et al. 2012) and South American leaf blight in rubber tree (Chee 1976). In addition, the optimal temperature for ascospores to form appressoria of about 30°C estimated in our study is within the temperature range from 16°C to 32°C in which *Phyllosticta* spp. ascospores are mostly released in South African orchards (Moyo et al. 2020), as well as corroborates the in vitro results from Kotzé (1963) that higher *Phyllosticta* sp. ascospore germination occurred at 29.5°C than at 15°C and 21°C–23°C.

The values of b_1 parameter demonstrated that, although the germ tube formation of both spore types occurred in the same range of temperatures, ascospore appressorium formation occurred in a wider range of temperatures than conidia, as observed by the near absence of conidial appressorium formation from 35°C onwards. Similar results with conidia were observed

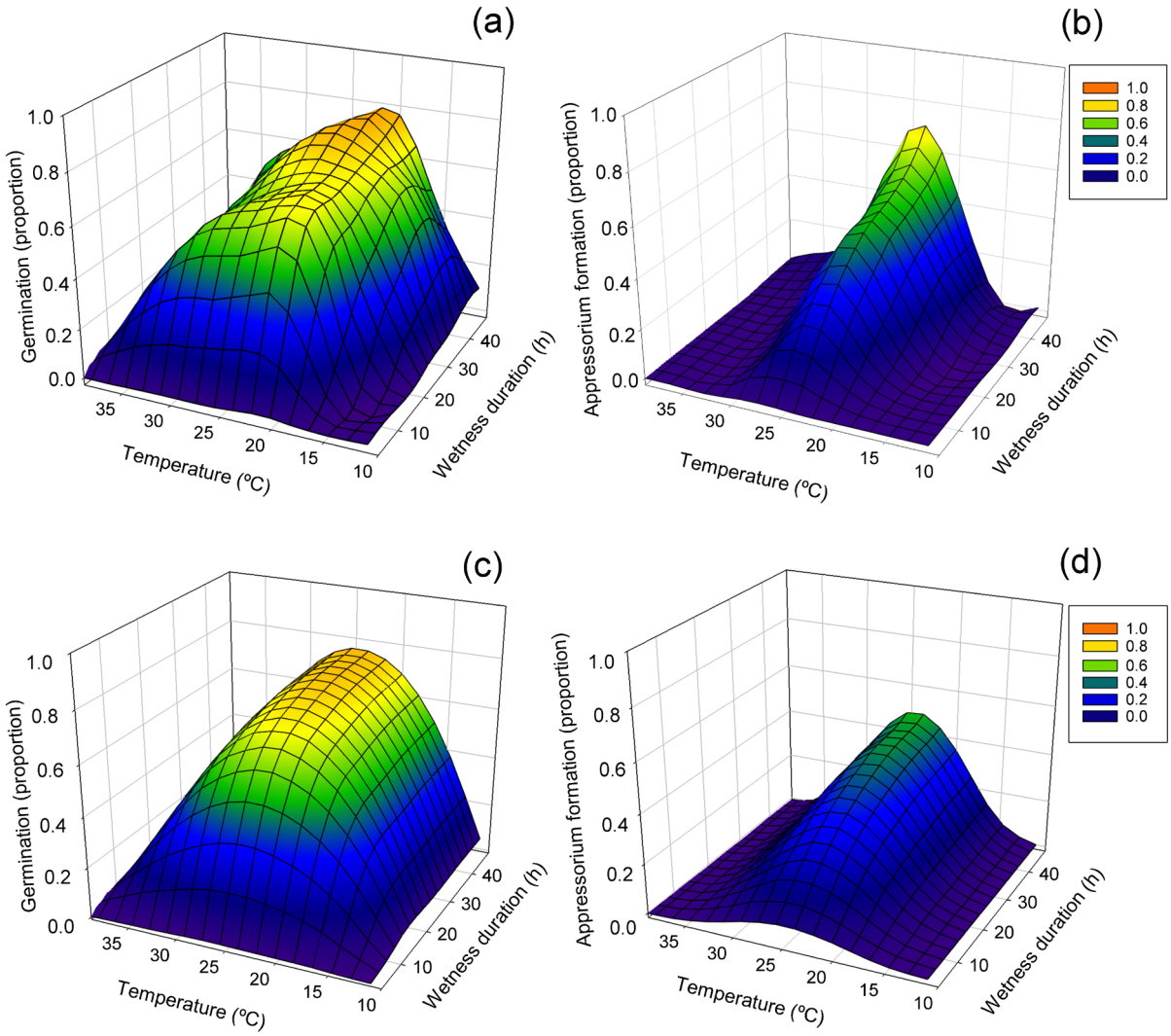

FIGURE 5 | Comparison of response surface curves with observed (a, b) and estimated (c, d) data using the beta-monomolecular model obtained from the average *Phyllosticta citricarpa* conidial in vitro germination (a, c), and appressorium formation (b, d) in proportion (*z*-axis) under diverse temperatures (*x*-axis) and wetness durations (*y*-axis). Pooled data from both experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

in studies with *P. citricarpa* (Korf 1998; Noronha 2002; Wang and Dewdney 2019). This may result in lower rates of infections from conidia in warmer climates because this specialised structure may be fundamental to *P. citricarpa* infection in the citrus tissues, as observed for *Phyllosticta ampelicida*, the causal agent of grape black rot, whose penetration peg penetrated an inert polymeric substratum only after appressorium formation (Shaw et al. 1998). The appressorium has been reported as necessary for tissue infections in other ascomycete-related pathosystems, such as rice blast (*Pyricularia grisea*) (Chumley and Valent 1990) and apple scab (*V. inaequalis*) (Steiner and Oerke 2007).

Ascospores formed fewer germ tubes and appressoria than conidia, even under the optimal temperatures. This difference in germination rate, even under similar conditions, observed for both *P. citricarpa* spores in our study was also found in previous studies with ascomycetous fungi (Gelain et al. 2024; Trapero-Casas and Kaiser 2007). In the case of *P. citricarpa*, this may be explained by differences in surface hydrophobicity and juice quality requirements for each spore, which are known only for conidia (Wang and Dewdney 2019). In addition, the site and collection time of both spores may have affected their viability. The conidia used in our assays were collected directly from the colonies with mucilage, which may produce germination self-inhibition compounds (Allen 1965; Dean 1997) removed after suspension preparation. In the case of ascospores, which were collected from the dish lids up to 21 days after crosses, these germination self-inhibition factors are probably lost after ejection, and some may have been ejected a few days before collection and lost their vigour to germinate, as observed in *Gibberella zeae* (Beyer and Verreet 2005). Another hypothesis is that the conidial suspensions were prepared using PDA mycelial disks. Thus, even removing PDA and pathogen fragments with cheesecloth, the nutritional conditions of the conidial suspension may be different compared to the ascospore suspensions. Further studies assessing ascospore germination need to consider that these factors may affect the pre-penetration process.

Our findings showing differences between environmental requirements for the germination of the two *P. citricarpa* spores

suggest that ascospores and conidia may have distinct and complementary roles in the CBS epidemiology. The critical period for *P. citricarpa* infections in the São Paulo citrus belt has been from October to March, and the average temperatures recorded in these months ranges from 23°C to 26°C (Fialho et al. 2024). The optimum temperature for germination and appressorium formation by conidia (~25°C) is within this range, while ~30°C found for maximum ascospore germination has been less frequent in this citrus-growing region, which may favour infections by conidia rather than ascospores.

The peaks of *P. citricarpa* ascospore germination and appressorium formation occurred mostly from 24 h of wetness onwards. This period is longer than 6–12 h found for other ascomycetous fungi, such as *Uncinula necator* (Gadoury and Pearson 1990), *Leptosphaeria maculans* (Huang et al. 2003), *G. zeae* (Beyer and Verreet 2005) and *V. inaequalis* (Steiner and Oerke 2007). In the case of conidial germination, the peaks in our study occurred mostly from 12 to 24 h of wetness. Wang and Dewdney (2019) found peaks after 12 h with a Floridian isolate tested at 24°C in a mixture of three parts of conidial suspension with one part of 2% orange juice, while Noronha (2002) observed conidium germination peaks within 24 h in a suspension containing sterile distilled water and sucrose for a Brazilian isolate. These variations in the length of the pre-penetration processes found for *P. citricarpa* may also be caused by the surface hydrophobicity and juice quality (Shaw et al. 1998, 2006; Wang and Dewdney 2019), as reported for conidia of other ascomycetous fungi such as *Colletotrichum graminicola* (Chaky et al. 2001) and *Botryosphaeriaceae* fungi (Sammonds et al. 2019). Therefore, because of these surface and nutritional effects on the *P. citricarpa* pre-penetration process, further studies need to be performed on the citrus fruit and leaf surfaces, as performed by Frare et al. (2019), to associate pre-penetration processes with the severity of CBS symptoms under different temperatures and wetness durations, establishing more accurate thresholds for predictive systems.

Although the assessments of CBS severity under weather-controlled conditions are necessary to obtain safer and more accurate predictive models, our data obtained in vitro, especially for ascospores, may contribute to improve the analysis of climate suitability for CBS in citrus-growing areas worldwide, as in the Mediterranean Basin, which has long been debated (Galvañ et al. 2022; Yonow et al. 2013). The models developed in these studies used data from Kotzé (1963), who estimated ascospore cardinal temperatures as 15°C (T_{min}), 27°C (T_{opt}) and 35°C (T_{max}) for ascospore germination. However, these temperatures were established before the development of molecular techniques for identifying *P. citricarpa*, and spores of the endophytic *Phyllosticta* species may have been used due to the morphological similarity with *P. citricarpa* (Baayen et al. 2002). In Tunisia, only conidia have been associated with infections (Ioos et al. 2024), and the model developed by Galvañ et al. (2022) for this inoculum showed that orchards in areas with a arid climate tend to be more affected by CBS than in areas with a Mediterranean climate.

The results presented here may contribute to a better understanding of the conditions required by each *P. citricarpa* inoculum type to germinate and form appressorium. Moreover, our models that describe the environmental requirements for the *P. citricarpa* pre-penetration process are preliminary data that could lead to the development of CBS predictive systems including both spores, or to map CBS risk areas worldwide based on their climate suitability after further refinement, impacting on the optimisation of this disease management and regulation rules in countries where CBS does not occur.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request, and in the Fundecitrus Research Data Repository (https://rdp.fundecitrus.com.br/).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.