

Effect of temperature and photoperiod on *Pseudomonas syringae* pv. *garcae* inoculum production

Júlia Marques Oliveira^{1,2} , Edson Ampélio Pozza¹ , Leônidas Leoni Belan³ ,
Marcelo Loran de Oliveira Freitas⁴ , Vitória Moreno Tedardi¹ 

¹Universidade Federal de Lavras, Departamento de Fitopatologia, Lavras, MG, Brasil

²Centro Universitário de Formiga, Formiga, Minas Gerais, Brasil

³Universidade Estadual da Região Tocantina do Maranhão, Centro de Ciências Agrárias, Imperatriz, Maranhão, Brasil

⁴Instituto Federal de Minas Gerais, Departamento de Ciências Agrárias, Bambuí, MG, Brasil

Contact authors: julia.moliveira@ymail.com; edsonpozza@gmail.com; leonidas.belan@uemasul.edu.br; marcelo.freitas@ifmg.edu.br; vitoria-mt@hotmail.com

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ABSTRACT

Bacterial halo blight (BHB), whose etiological agent is the bacterium *Pseudomonas syringae* pv. *garcae*, is one of the main diseases occurring in coffee (*Coffea arabica* L.) leading to losses in nurseries and fields. This study aimed to assess the effect of five temperatures (20, 25, 30, 35, and 40 °C) and three photoperiods (continuous light, 12-hour light/dark, continuous dark) on the *P. syringae* pv. *garcae* inoculum concentration and the BHB progression resulted from this inoculum, in coffee seedlings. Using a factorial design (5 x 3) with four replicates, Petri dishes inoculated with *P. syringae* pv. *garcae* were incubated for 48 hours, and the concentrations and bacterial cell dimensions were measured. The resulting inoculum was then used to inoculate coffee seedlings at 23 ± 2 °C in one leaf pair by wounding inoculation using a multi-needle apparatus. The disease severity was assessed, and the area under the disease progress curve (AUDPC) was calculated. Significant interaction ($p < 0.05$) between temperature and photoperiod was observed for inoculum concentration and cell dimensions. The highest inoculum concentration (2.2×10^9 CFU/ml) was recorded at 25 °C under continuous light. The largest bacterial cells (4.4 µm in length and 0.7 µm in width) resulted from temperatures of 30 °C with 12-hour light/dark. For the AUDPC of BHB, a difference ($p < 0.05$) was observed only between inoculum production temperatures. The highest AUDPC was recorded at 25 °C, with a gradual decline observed as the temperature increased.

Key words: Bacterial inoculation; bacterial halo blight; *Coffea arabica* L.; concentration; disease severity.

1 INTRODUCTION

Coffee is one of the most important commodities in the global market, with *Coffea arabica* and *Coffea canephora* being the predominant species under cultivation. Brazil is the main producer and exporter, accounting for 39% of the global coffee production in 2023 (United States Department of Agriculture - USDA, 2024). Projections for 2024 estimate Brazil's coffee production at 58.8 million 60 kg bags of green bean equivalent, with the state of Minas Gerais contributing 68% of the national *C. arabica* production (Companhia Nacional de Abastecimento - CONAB, 2024).

However, several factors have reduced the productivity of coffee plantations, including climatic adversities, pests, and diseases. Fungi, viruses, bacteria, and nematodes are examples of etiological agents of coffee diseases. Among these, the bacterium *Pseudomonas syringae* pv. *garcae* (Amaral; Teixeira; Pinheiro, 1956) stands out for causing bacterial halo blight (BHB). This disease is prevalent in seedling nurseries and crop fields across key coffee-producing regions. It spreads quickly, leading to severe defoliation, branch desiccation, and difficult control efforts. The high disease progression rate results in significant crop losses, from the early nursery stages to mature fields, making management challenging and often costly for producers. The BHB is particularly evident in

regions over 900 m altitude with a temperature close to 20 °C, exposed to the wind, and a leaf wetness duration of more than 4 hours or where the crop is mechanized harvesting (Pozza et al., 2022).

The bacterium mainly infects new tissues, such as seedlings in nurseries, new plants in the field, and expanding leaves near the apical meristem during the production phase, in addition to causing rot in coffee branches and fruits (Rodrigues et al., 2013). In the leaves, the initially observed symptoms are small irregular lesions with a soggy appearance, with subsequent tissue necrosis or blight. This necrotic area may increase in size, and yellow halos may appear around it, explaining the name of the disease. The lesions can also coalesce and break the leaf blade (Belan et al., 2014b).

The development of lesions, their coalescence, and the extent of the yellow halo are influenced by the coffee plant's resistance and specific environmental conditions. Factors such as temperature, photoperiod, and other environmental variables play crucial roles in affecting both the pathogen and the host, as well as their interaction, either promoting or hindering the onset and progression of the disease (Silva et al., 2016). Environmental factors can determine the geographic distribution, incidence, and severity of disease and, in many cases, are specific to the pathogen in question. Among these factors, temperature is most frequently correlated with disease

epidemiology, followed by humidity and light (Colhoun, 1973; Campbell; Madden, 1990).

In the field, temperature and luminosity oscillate daily, but when these factors remain constant under artificial conditions, it is possible to evaluate their impact on pathogen biology (Andrade et al., 2007). Knowing the effect of environmental factors *in vitro* and the infectivity of pathogens is essential for producing inocula and determining the ideal conditions for artificial inoculation (Mueller; Buck, 2003). Because disease occurrence depends on environmental factors, the morphology, and physiology of the pathogen are also influenced by environmental conditions, which can alter the infectious capacity of the pathogen and consequently the intensity of the disease. Therefore, the temperature and photoperiod during the incubation period can alter the morphological characteristics and therefore the infectivity of *P. syringae* pv. *garcae*, in addition to its inoculum production.

Temperature is essential in the multiplication of *P. syringae* pv. *garcae* by influencing the activity rates of various enzymes. However, the specific expression of genes or the activity of enzymes can vary within the species. For *P. syringae* pv. *garcae*, genes, and enzymes responsible for toxin synthesis and flagellum motility are suppressed at 30°C. In contrast, enzymes associated with nutrient transport and protein translation are activated at this same temperature, reflecting a shift in the pathogen's metabolic priorities in response to environmental conditions (Hockett; Burch; Lindow, 2013). Multiplication of *P. syringae* pv. *garcae* *in vitro* was performed at 28 °C in several studies (Freitas et al., 2022, Raimundi et al., 2021, Pérez et al., 2017; Rodrigues et al., 2017; Ithiru et al., 2013). Oliveira et al. (2024) assessed the efficiency of different *P. syringae* pv. *garcae* inoculation methods to enhance the highest BHB intensity. According to the same author, wounding inoculation by injection and multi-needle resulted in a higher area under the incidence progress curve (AUIPC) and area under the severity progress curve (AUSPC) values for BHB than inoculation without wounding. However, it is uncertain which temperature range in the inoculum production leads to the greatest *P. syringae* pv. *garcae* concentration and its relationship with luminosity.

Additionally, temperature and photoperiod are environmental factors that can significantly impact bacterial cell morphology, leading to alterations in cell size, shape, and structure. These morphological adaptations can greatly influence bacterial growth, survival, and pathogenicity (Lam; Wheeler; Tang, 2014). Understanding how these factors influence cell morphology is crucial for advancing knowledge of bacterial ecology, physiology, and pathogenicity. Additionally, such insights are fundamental for developing effective management strategies for BHB, as environmental conditions directly impact the pathogen's ability to cause disease. Therefore, this study aimed to assess the effects of

different temperatures and photoperiods on the *P. syringae* pv. *garcae* inoculum production and the BHB severity in coffee seedlings caused by these inocula.

2 MATERIAL AND METHODS

The experiment was performed in two stages, *in vitro* and *in vivo*, and repeated twice.

2.1 Effect of temperature and photoperiod on the *P. syringae* pv. *garcae* inoculum concentration and cell dimensions

The bacterial isolate used in this study was derived from the *P. syringae* pv. *garcae* pathotype 'souche' (CIRM, plant-associated bacteria; CFBP1634). The inoculum preparation and pathogen cultivation were performed in 523 media, following the protocol described by Kado and Heskett (1970).

From pure colonies, a suspension with a concentration of 1×10^8 CFU/ml was prepared and calibrated in a spectrophotometer according to the method described below to determine the inoculum concentration. A total of 100 µL of this suspension was pipetted into Petri dishes containing MB1 medium, spread evenly with a Drigalski spatula, and closed with plastic film.

Five temperatures, 20, 25, 30, 35, and 40 °C, and three light exposures (photoperiods) were evaluated in a factorial (5 x 3) design, totaling 15 treatments. A randomized block design with four replicates was used, i.e., with 60 plates in total. The plates were kept in BODs for 48 hours and exposed to continuous light, 12-hour light/dark, or continuous dark. The absence of light was achieved by wrapping the plates with aluminum foil.

After 48 hours, the inoculum concentration was determined in a spectrophotometer at an optical density of 600 nm. Ten milliliters of 0.85% saline solution were added to each Petri dish. Next, a fraction of this suspension was pipetted into an ELISA plate well. The absorbance value was then measured in each treatment. This value was converted into an inoculum concentration following Oliveira and Romeiro (1990):

$$IC = 600/0.028 + [1.516 * (10^{-10})]$$

Where:

IC = inoculum concentration (CFU/ml)

To measure the size of *P. syringae* pv. *garcae* cells from the bacterial suspensions described above, the suspensions were scraped onto slides, which were then covered with coverslips for immediate reading. The slides were observed under a Zeiss Axio Observer Z1® microscope at 1000x magnification. Images of the bacterial cells were obtained, and the width and length of 30 cells per treatment were measured using AxioVision® software.

2.2 Pathogenicity test in seedlings *in vivo*

The inoculum suspensions obtained for each treatment in the previous step were used to inoculate healthy seedlings of *C. arabica* var. Catuaí Vermelho IAC-99, which is susceptible to the pathogen. These seedlings had three to four pairs of true leaves and were grown in polyethylene bags 0.065 m in diameter and 0.20 m in height. The growth substrate consisted of soil, cattle manure, and sand at a ratio of 2:1:1.

Inoculation was performed with the suspensions and corresponding inoculum concentrations obtained in the same treatments and following the same experimental design as those described in the *in vitro* experiment. Thus, the first pair of fully developed leaves from the apex of the plant was inoculated in each of the 60 seedlings. The pathogen was inoculated via wounds using a multi-needle set immersed in the inoculum suspension (Oliveira et al., 2024). The controls were inoculated with a sterile saline solution. After inoculation, the seedlings were kept in a humid chamber for 24 hours in a controlled environment at 23 ± 2 °C with a photoperiod of 12 hours. They received localized daily irrigation as needed so as not to wet the leaves.

The evaluation of BHB severity began 48 hours after inoculation and was evaluated every two days, only in the two inoculated leaves, totalizing ten evaluations. The severity was estimated with the aid of a diagrammatic scale for BHB proposed by Belan et al. (2014a). The severity values from the graded scale were integrated over time to obtain AUDPC, according to the equation proposed by Shaner and Finney (1977).

2.3 Statistical analysis

Firstly, the assumptions of the analysis of variance were checked. To assess normality and homoscedasticity, the Shapiro-Wilk and Bartlett tests were used, respectively. The data followed the assumptions of the analysis of variance and did not need to be transformed. A joint analysis was performed for the experiments repeated over time. For both experiments, the analysis of variance followed a 5 x 3 factorial arrangement with a randomized block design, with factor 1 comprised of five temperatures such as 20, 25, 30, 35, and 40 °C, and factor 2 with three photoperiods, namely, continuous light, 12-hour light/dark and continuous dark, totaling 15 treatments.

When the F test ($p < 0.05$) was significant for photoperiod, Tukey's test of means was performed ($p < 0.05$). For the temperature variable, linear regression models were fitted for both inoculum concentrations *in vitro* and AUDPC *in vivo*. Linear regression models were also fitted for the variable bacterial cell size (length and width). The models were selected based on the significance of the t-test of the parameters of the fitted regression models ($p < 0.05$), the highest coefficient of determination (R^2), and the smallest mean square of the deviations obtained in the analysis of variance.

The maximum points of the mean concentration (10^9 CFU/ml), length (μm), and width (μm) variables were obtained by equalizing the fitted regression models to zero, and then their first-order derivative was calculated. These statistical analyses were performed in R software (version 3.4.2).

Pearson correlation analysis ($p < 0.05$) was carried out using R software (version 3.4.2) to verify the correlations among temperatures, AUDPC, inoculum concentrations, and bacterial cell length and width.

3 RESULTS

There was no difference in the joint analysis of the experiments repeated in time for either the *in vitro* or *in vivo* experiments. Thus, the following data correspond to the means of the two experiments.

3.1 *P. syringae* pv. *garcae* inoculum concentration and cell size under different temperatures and photoperiods

The interaction between photoperiod and temperature significantly affected bacterial inoculum concentration (CFU/ml) ($p < 0.05$). The highest mean inoculum concentration of 1.22×10^9 CFU/ml was obtained under the 12-hour light/dark photoperiod (Figure 1).

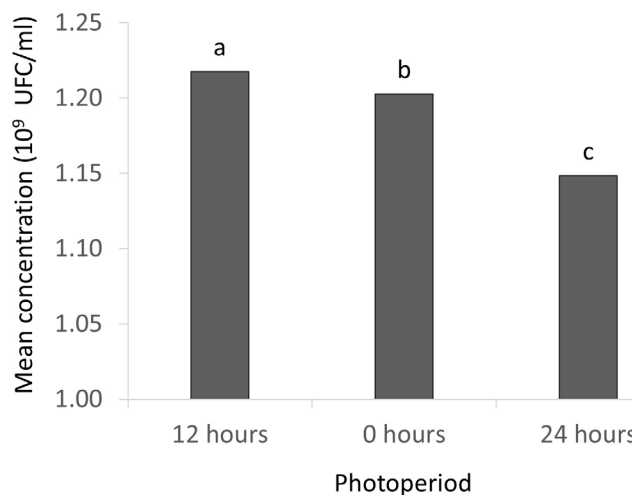


Figure 1: Mean inoculum concentration of *Pseudomonas syringae* pv. *garcae* (10^9 CFU/ml) under different photoperiods. Bars followed by the same letter do not differ by Tukey's test ($p < 0.05$).

The effect of temperature on inoculum was adjusted by third-degree regressions, estimating the highest inoculum concentration, which was 2.2×10^9 CFU/ml, observed at 25 °C under a 24-hour light photoperiod (Figure 2). At temperatures of 35 °C and above, bacterial growth was nearly zero across all three photoperiods.

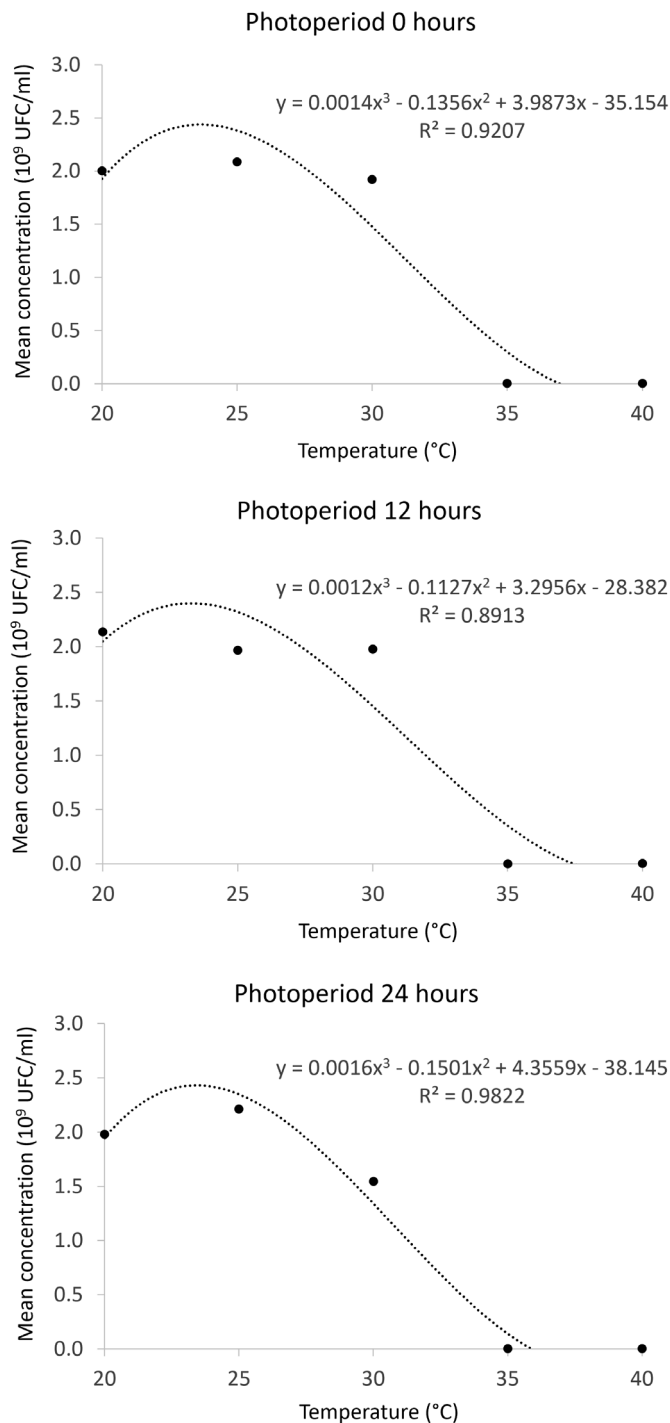


Figure 2: Mean inoculum concentration of *Pseudomonas syringae* pv. *garcae* (10^9 CFU/ml) as a function of temperature under different photoperiods (0, 12, and 24 hours of light).

There was an interaction ($p < 0.05$) between photoperiod and temperature on the dimensions of *P. syringae* pv. *garcae* cells. The longest mean length (2.41 μm) and mean width (0.38 μm) of the bacterial cells were observed under photoperiods of 0 hours and 24 hours of light (Figure 3).

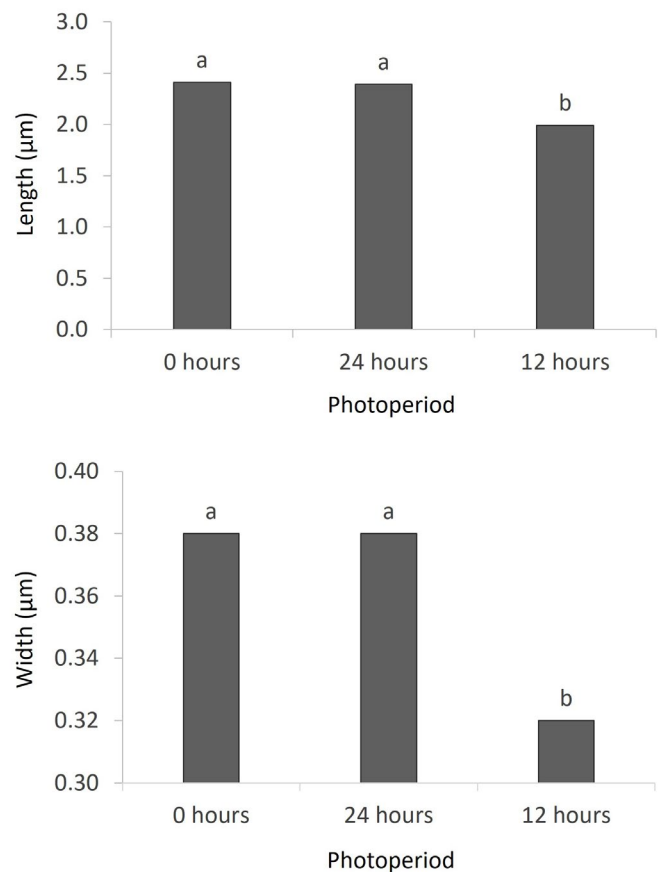


Figure 3: Length and width of *Pseudomonas syringae* pv. *garcae* cells under different photoperiods. Bars followed by the same letter do not differ by Tukey's test ($p < 0.05$).

At 30 $^{\circ}\text{C}$, the 12-hour light/dark resulted in the largest bacterial cells, with dimensions of 4.4 μm in length and 0.7 μm in width. No bacterial growth was detected at 40 $^{\circ}\text{C}$, while at 35 $^{\circ}\text{C}$, growth occurred only under continuous dark and continuous light conditions (Figure 4).

3.2 Influence of inoculum production conditions on BHB severity in coffee seedlings inoculated with *P. syringae* pv. *garcae*

The highest BHB severity, reaching 4.9 %, was observed 20 days after inoculation (dai) with inoculum produced under a 12-hour light/dark photoperiod at 25 $^{\circ}\text{C}$ (Figure 5). In contrast, the lowest severity (0 %) across all photoperiods occurred two dai when the inoculum was cultivated at 30 $^{\circ}\text{C}$.

For the AUDPC of BHB, there was a difference ($p < 0.05$) between inoculum production temperatures. The highest AUDPC occurred when the inoculum was produced in the range of 20 to 30 $^{\circ}\text{C}$, with the maximum recorded at 25 $^{\circ}\text{C}$, and a gradual decline was observed as the temperature increased. As expected, bacterial cells cultivated at 35 and 40 $^{\circ}\text{C}$ did not result in any symptoms in coffee seedlings, as bacterial multiplication did not occur at this temperature (Figure 6).

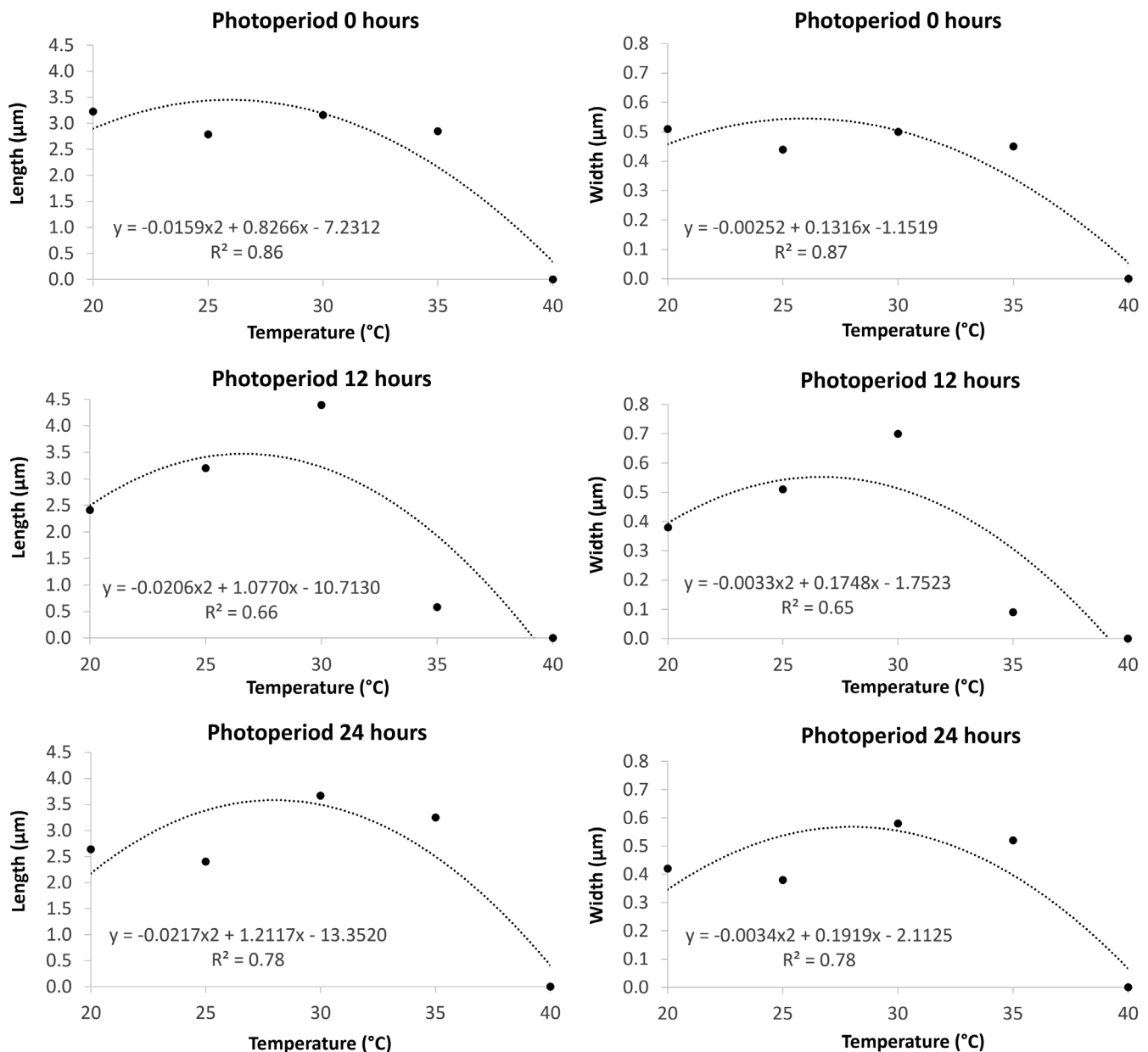


Figure 4: Length and width of *Pseudomonas syringae* pv. *garcae* bacterial cells as a function of temperature under different photoperiods (0, 12, and 24 hours of light).

There was a significant ($p < 0.05$) negative correlation between the *in vitro* incubation temperature and AUDPC (-0.73) and between temperature and inoculum concentration (-0.89) (Table 1). Conversely, there was a significant ($p < 0.05$) positive correlation between AUDPC and inoculum concentration variables. In this case, the coefficient was also high (0.82); that is, the higher the inoculum concentration, the higher the AUDPC.

4 DISCUSSIONS

Despite the importance of bacterial diseases, there is limited information in the existing literature concerning

the thermal ranges and light regimes conducive to their reproduction. Determining the environmental factors essential for *in vitro* cultivation and plant infection is crucial to supporting artificial inoculation (Mueller; Buck, 2003). In studies involving *P. syringae* pv. *garcae*, Belan et al. (2016) prepared the inoculum at 28 °C with a 24-hour light photoperiod, while Freitas et al. (2022) utilized a 12-hour photoperiod at the same temperature. In contrast, Beriam et al. (2017), Domingues et al. (2015), and Rodrigues et al. (2016), conducted their incubations at 28 °C without photoperiod. Moreover, the use of different photoperiods for other *Pseudomonas* genera has been described in the

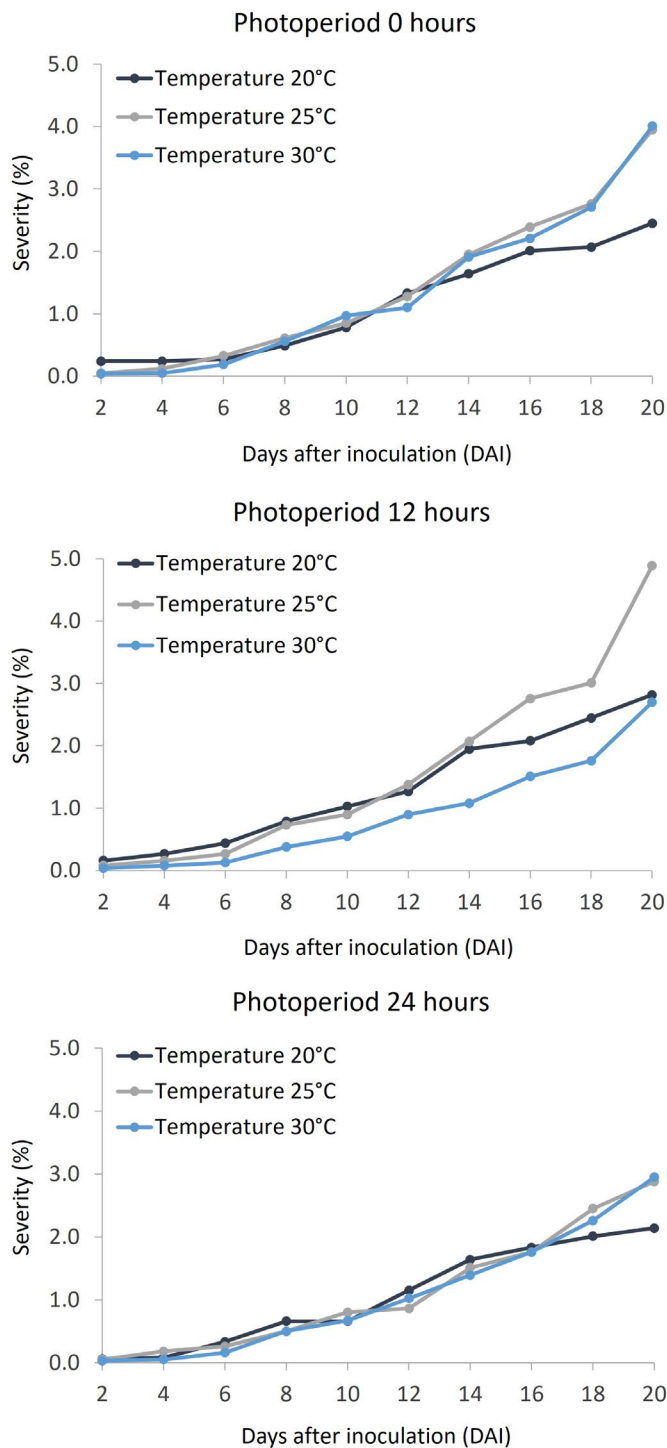


Figure 5: Progress of severity (%) of bacterial halo blight in coffee seedlings (*Coffea arabica* L.) inoculated with *Pseudomonas syringae* pv. *garcae* suspensions produced under temperatures of 20, 25, and 30°C, and photoperiods of 0, 12, and 24 hours of light.

literature. Bodelier et al. (1997) studied the effect of 14- and 20-hour photoperiods in the *Pseudomonas chlororaphis* growth. According to the authors, the 20-hour light caused an increase in *P. chlororaphis* growth. Therefore, it is essential

to standardize the optimal temperatures and photoperiods for inoculum production to effectively induce BHB symptoms.

Additionally, the influence of temperature on *in vitro* growth has also been observed in various *Pseudomonas syringae* pathovars. For example, Young, Luketina and Marshall (1977) reported a stable growth rate for *P. syringae* within the temperature range of 23 to 33 °C, indicating no significant alteration in bacterial growth within this range. Nüske and Ritsche (1989) related that *P. syringae* pv. *phaseolicola* exhibited an optimal growth rate at 27 °C under the same incubation conditions. Similarly, in our study, the optimal conditions for cultivating *P. syringae* pv. *garcae* and achieving the highest inoculum concentration were found to be at a temperature of 25 °C combined with a continuous 24-hour light photoperiod.

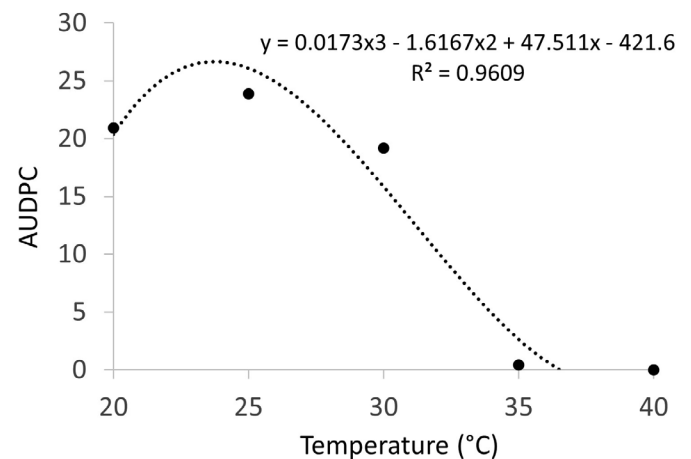


Figure 6: The area under the disease progress curve (AUDPC) of bacterial halo blight (*Pseudomonas syringae* pv. *garcae*) in coffee seedlings (*Coffea arabica* L.) with wounding inoculation.

Table 1: Correlation coefficients between the variables temperature (°C), AUDPC, inoculum concentration (CFU/ml), length (μm), and width (μm) of bacterial cells.

	Temperature	AUDPC	Concentration	Length	Width
Temperature	ns	-0.73*	-0.89*	-0.62*	-0.62*
AUDPC	-0.73*	ns	0.82*	0.55*	0.55*
Concentration	-0.89*	0.82*	ns	0.67*	0.66*
Length	-0.62*	0.55*	0.67*	ns	1.00*
Width	-0.62*	0.55*	0.66*	1.00*	ns

* Pearson correlation ($p < 0.05$); ns = not significant.

In previous studies, various concentrations of *P. syringae* pv. *garcae* suspension has been successfully employed to induce symptoms post-inoculation. Notable concentrations include 10^9 CFU/mL (Ithiru et al., 1970; Belan et al., 2016), 10^8 CFU/mL (Ithiru et al., 2013), and 3×10^8 CFU/mL (Rodrigues et al., 2017). However, the *P. syringae*

pv. *garcae* concentration of 1.22×10^9 CFU/mL obtained in this study aligns closely with the 1.6×10^9 CFU/mL reported by Oliveira et al. (2024).

The photoperiod during inoculum production did not significantly affect the AUDPC, as the concentration was sufficient to cause infection across all three photoperiods studied. In contrast, the temperature during inoculum production had a notable impact on AUDPC, primarily due to its influence on inoculum concentration. The highest disease severity was observed with inoculum produced at 25 °C, while the lowest severity occurred with inoculum produced at 30 °C.

Bacteria of the genus *Pseudomonas* typically range from 1.5 to 5.0 µm in length and 0.5 to 1.0 µm in width (Krieg, 1984). In this study, a temperature of 30 °C combined with a 12-hour light photoperiod produced the largest *P. syringae* pv. *garcae* cells. The dimensions of 4.4 µm in length and 0.7 µm in width, fit within the expected size range.

Although the largest cell size was observed at 30°C, the inoculum produced at 25°C proved to be more effective in inducing BHB symptoms in coffee seedlings. While larger bacterial cells might be more effective at colonizing large wounds or damaged tissues, their size could limit their ability to navigate through narrower spaces, potentially restricting their spread within the plant (Yang; Blair; Salama, 2016). Therefore, the temperature is an important factor that influences bacterial cell size and pathogenicity.

Bacteria can recognize changes in local temperature through multiple mechanisms, including the alteration of DNA topology, RNA structuring and metabolism, and protein activity and processing. The histone-like nucleoid structuring protein, H-NS, present in several Gram-negative pathogens, can repress the expression of virulence genes. At low temperatures, the DNA in the promoter region of certain virulence genes adopts a bent conformation, stabilized by H-NS, which binds to curved DNA at A:T sites, blocking RNA polymerase access to the promoter. At higher temperatures, this homotypic H-NS binding is reduced as the DNA bend relaxes, allowing RNA polymerase access to the promoter. This reduction in RNA polymerase binding at the promoter leads to decreased transcription of genes encoding virulence factors when the bacterium is outside the host (Lam; Wheeler; Tang, 2014).

The negative correlation between temperature and AUDPC, and between temperature and inoculum concentration (CFU/ml), indicated that the lower the temperature, the greater the inoculum concentration, and the highest disease severity. In the study carried out by Young, LUKETINA AND MARSHALL (1977), the growth rate of *P. syringae* fell dramatically at temperatures above 33 °C. Nevertheless, in the same study, all species of bacteria of the *Pseudomonas* genus were tolerant to cold, i.e., psychrotrophic, an important factor explaining the survival of the pathogen and the occurrence of the disease at mild temperatures.

There is little information available in the literature regarding correlations among these factors concerning bacterial diseases. In pathosystems involving certain fungi, such as the genus *Botrytis*, a negative correlation is common; i.e., an increased inoculum concentration and disease severity are observed as a function of decreased temperature (Latorre et al., 2002; Nair; Allen, 1993; Sirjusingh; Sutton, 1996). Conversely, for the AUDPC and inoculum concentration (CFU/ml) variables, there was a significant positive correlation with an R^2 of 0.82; that is, the higher the inoculum concentration was, the greater the disease severity. According to Silveira et al. (2003), the severity of melon fruit blotch increased with increasing inoculum concentration of *Acidovorax avenae* subsp. *citrulli*.

5 CONCLUSIONS

The highest *P. syringae* pv. *garcae* inoculum concentration was achieved at 25 °C under a 24-hour light photoperiod. While larger bacterial cell sizes were recorded at 30°C with a 12-hour light/dark cycle, the inoculum produced at 25°C led to the highest AUDPC values, regardless of the photoperiod. These results highlight the critical role of temperature in optimizing bacterial growth and enhancing disease severity, indicating that the *P. syringae* pv. *garcae* inoculum production at 25°C is the most effective for inducing BHB, with photoperiod playing a less significant role in this aspect.

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7 AUTHOR'S CONTRIBUTIONS

Conceptual Idea: Pozza, E. A.; Oliveira, J. M.; Belan, L. L, Methodology design: Pozza, E. A.; Oliveira, J. M.; Belan, L. L, Data collection: Oliveira, J. M.; Belan, L. L, Freitas; M. L. O., Data analysis and interpretation: Pozza, E. A.; Oliveira, J. M.; Belan, L. L; Freitas, M. L. O.; Tedardi, V. M., and Writing and editing: Oliveira, J. M.; Tedardi, V. M.

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