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Factors influencing *in vitro Chrysoporthe cubensis* development and first-time reproduction of wilting symptoms in eucalypt

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Magister Scientiae

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Dissertation submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Gleiber Quintao Furtado

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ABSTRACT

SILVA, Eduarda de Vasconcelos, M.Sc., Universidade Federal de Viçosa, July, 2025. **Factors influencing *in vitro Chrysoporthe cubensis* development and first-time reproduction of wilting symptoms in eucalypt**. Adviser: Gleiber Quintao Furtado.

The fungus *Chrysoporthe cubensis* was reported to be associated with mini-stumps of different clones of the hybrid *Eucalyptus grandis* × *Eucalyptus urophyll*a, initially causing wilting, which later progressed to dieback of the aerial parts and plant death in mini-clonal hedges. This study aimed to reproduce wilting symptoms caused by *C. cubensis* in eucalyptus plants and to evaluate the influence of various factors on the *in vitro* development of *C. cubensis*. Conidial production by *C. cubensis* varied depending on the combination of culture medium and temperature. The highest sporulation occurred on PDA and PCA media containing eucalyptus stem fragments on the surface, incubated at 28°C. In the absence of eucalyptus fragments, greater sporulation was observed on PCA at 25°C and on PDA at 28°C. The most favorable condition for mycelial growth was PDA at both 25°C and 28°C. Light regime was not considered a limiting factor for *in vitro* conidial germination. After 12 hours of incubation period, higher germination occurred under continuous light. However, no significant difference in germination rates was observed after 24 hours of incubation, regardless of the light regime. Regarding the influence of temperature and incubation period, conidial germination increased with longer incubation periods at 25°C and 28°C. For the first time, symptoms associated with *Chrysoporthe*-induced wilting were successfully reproduced in eucalypt plants. Wilting and necrotic lesions inside the branches were observed exclusively in plants pruned with shears, both those previously immersed in a conidial suspension and those pruned and subsequently sprayed with the suspension. These results provide new insights into the *C. cubensis*-eucalypt pathosystem and highlight the role of wounds caused by periodic pruning of mini-stumps as entry points for the pathogen.

Keywords: Chrysoporthe wilt; mini-stumps; pruning wounds

RESUMO

SILVA, Eduarda de Vasconcelos, M.Sc., Universidade Federal de Viçosa, julho de 2025. **Fatores influenciando desenvolvimento de *Chrysoporthe cubensis in vitro* e reprodução inédita dos sintomas de murcha em eucalipto**. Orientador: Gleiber Quintao Furtado.

O fungo *Chrysoporthe cubensis* foi relatado associado a minicepas de diferentes clones de eucalipto do híbrido *Eucalyptus grandis* × *Eucalyptus urophylla*, causando inicialmente murcha, que posteriormente evoluiu para o secamento da parte aérea e morte, em minijardim clonal. Os objetivos deste estudo foram reproduzir o sintoma de murcha causado por *C. cubensis* em plantas de eucalipto e avaliar a influência de fatores no desenvolvimento *in vitro*. A produção de conídio de *C. cubensis* variou em função das diferentes combinações de meio de cultura e temperatura. Os meios BDA e BCA contendo fragmento de haste de eucalipto na superfície do meio, proporcionou maior esporulação sob 28°C. Na ausência de fragmento de eucalipto, *C. cubensis* apresentou maior esporulação em BCA em 25°C e BDA em 28°C. A melhor condição para o crescimento micelial ocorreu em BDA em 25°C e 28°C. O regime de luz não foi considerado um fator limitante para a germinação de conídios in vitro. Após 12 horas de período de incubação, maior germinação ocorreu sob luz contínua. Porém, não houve diferença na taxa de germinação com 24 horas de incubação, independente do regime de luz. Para a germinação sob influência da temperatura e período de incubação, foi observado um aumento na sua taxa com a extensão do período de incubação em 25 e 28°C. Pela primeira vez foi reproduzido os sintomas de murcha causado por *C. cubensis* em plantas de eucalipto. Os sintomas, como murcha e lesões necróticas no interior dos ramos, foram observados exclusivamente em plantas podadas com tesoura, tanto naquelas previamente imersas em suspensão de conídios quanto naquelas podadas e atomizadas com a suspensão. Estes resultados trazem novos conhecimentos sobre o patossistema *C. cubensis*-eucalipto e sobre o papel dos ferimentos decorrentes das podas periódicas em minicepas como portas de entrada para o patógeno.

Palavras-chave: murcha de Chrysoporthe; mini-cepas; ferimento por poda

SUMMARY

1. INTRODUCTION

Eucalypt (*Eucalyptus* spp.) is one of the main forest cultures planted in Brazil, covering approximately 7.8 million hectares, which corresponds to 76% of the total area of planted forests in the country. Plantations are predominantly concentrated in the Southeast region, with the state of Minas Gerais accounting for approximately 63% of the cultivated area in that region. In 2023, the average productivity was estimated at 33.7 $m^3\ ha^{-1}\ year^{-1}$, with an average harvest cycle of 7.2 years (Ibá, 2024). Although eucalypt is well adapted to the diverse environmental conditions in Brazil, numerous diseases pose a threat to its development and productivity, both in nursery and in the field (Alfenas et al, 2004). For instance, the eucalypt canker, caused by *Chrysoporthe cubensis* (Bruner) Gryzenhout. & M.J.Wingfield, was regarded as the most limiting disease for the establishment of plantation in Brazil (Ferreira, 1989; Ferreira and Milani, 2004). This pathogen has a broad geographic distribution and has been reported in countries across South and Central America, Africa, Southeast Asia, and Oceania (Oliveira et al., 2021; Pegg et al., 2010; Gryzenhout et al., 2004; Nakabonge et al., 2006; Sharma et al., 1895; Wingfield et al., 1989; Hodges et al., 1986).

The causal agent of canker, a member of the family Cryphonectriaceae, was first reported in Cuba as *Diaporthe cubensis* (Bruner, 1917). In Brazil, its occurrence was first recorded in 1973, marking one of the most damaging diseases in eucalypt plantation during the 1970s (Ferreira, 1989). Later, based on morphological and cultural characteristics, the species was renamed *Cryphonectria cubensis* (Hodges,1980). However, due to morphological and molecular differences from other species of *Cryphonectria*, the establishment of a new genus, *Chrysoporthe*, was proposed specially to accommodate this species, which was then officially renamed *C. cubensis* (Gryzenhout et al., 2004). In young plants, canker disease symptoms consist of stem girdling that leads to vascular cambium death, whereas in more mature individuals are observed swellings, cracks, breakage, and cankers on the tree stem (Ferreira, 1989).

Chrysoporthe cubensis forms two distinct types of fruiting bodies. Pycnidia are the first to develop and are found in greater abundance on the upper portions of the stem. These structures are erumpent to superficial, pear-shaped with a well-defined neck, and exhibit a brownish-reddish coloration. These conidia adhere to each other forming cirrhi, which resemble orange-colored cords (Gryzenhout et al., 2004; Ferreira, 1989). Perithecia occurs predominantly at the base of the stem, being observed in lower quantities compared to pycnidia. They are generally

semi-immersed in the host tissues and contain unitunicate asci with fusoid to ellipsoid shapes (Gryzenhout et al., 2004; Hodges, 1980).

Recently, *C. cubensis* was found associated with eucalypt mini-stumps in a mini-clonal hedge, exhibiting symptoms distinct from those previously described for eucalypt canker. The symptomatic mini-stumps showed wilting that progressed to partial or complete canopy drying, followed by the death of the mini-stumps. In addition, necrotic lesions were observed in the inner stem tissues (Martins et al., 2023). The authors were able to confirm the pathogenicity of the isolates using six eucalypt clones, all clones showed necrotic lesions in the xylem of eucalypt rooted mini-cuttings inoculated in the stem with mycelial plugs of the pathogen. Interestingly, wilting and other external symptoms were not observed. Thus, reproducing wilt symptoms under controlled conditions using alternative inoculation procedures should be attempted again, as this information could help clarify how the pathogen infects mini-stumps in mini-clonal hedges and support the development of effective control strategies. Even though *C. cubensis* is a well-known pathogen, there is few information on the optimal conditions for inoculum production of the pathogen and epidemiological aspects of the disease.

Accordingly, the objectives of this study were: (i) to determine the influence of temperature and culture medium on conidial sporulation and mycelial growth of *C. cubensis*; (ii) evaluate the effect of luminosity, incubation period, and temperature on germination of conidia of *C. cubensis in vitro*; and (iii) reproduce wilting symptoms caused of *C. cubensis* in eucalypt plants using different inoculation procedures.

2. MATERIALS AND METHODS

2.1 Isolate of *C. cubensis*

Chrysoporthe cubensis isolate (COAD3401) used in this study was obtained from a eucalypt mini-stump, hybrid *Eucalyptus urophylla* x *Eucalyptus grandis*, showing wilting symptom, and identified through phylogenetic analysis (Martins et al., 2023). The isolate was preserved and stored in the Octavio de Almeida Drumond Collection (COAD) at the Universidade Federal de Viçosa (UFV). It was grown on PDA medium (potato dextrose agar) at 25°C for five days.

2.2 Conidial sporulation of *C. cubensis*

In order to optimize the conidial sporulation of *C. cubensis*, the effects of culture medium and temperature were evaluated in two separate experiments. Both experiments were performed

similarly, except that in one experiment a sterilized, autoclaved 2.5-cm eucalyptus stem fragment (clone 200000837) was placed on the surface of the medium in each Petri dish. The culture media used were potato dextrose agar (PDA) (39 g of synthetic Kasvi® medium, 5 g of agar, chloramphenicol 100 $mg.l^{-1}$, 1000 ml of deionized water), potato carrot agar (PCA) (20 g of potato extract, 20 g of carrot extract, 20 g of agar, chloramphenicol 100 $mg.l^{-1}$, 1000 ml of deionized water), and oat meal agar (OMA) (40 g of oat meal, 20 g of agar, chloramphenicol 100 $mg.l^{-1}$, 1000 ml of deionized water). After preparation, all media were autoclaved at 121°C for 20 minutes. A 5-mm-diameter mycelial disk from a 7-day-old culture of *C. cubensis* was taken from the edge of the colony and transferred to Petri dishes (90×15 mm) containing 20 mL of each culture medium. The plates were kept at 25 ± 1°C and 28 ± 1°C under a 12-hour photoperiod. To evaluate the conidial sporulation, 30 mL of sterilized deionized water were added to the surface of 13-day-old colonies. Conidia were removed by scraping with a Drigalski handle, and the suspension was filtered through a double layer of gauze and quantified using a hemacytometer (Alfenas & Mafia, 2016).

The experiments were conducted in a completely randomized design in a 3 (media) × 2 (temperatures) factorial scheme, with five replicates. These experiments were repeated once. Data from both experiments were transformed using Log_{10} and subjected to analysis of variance followed by Tukey's test at a 5% probability level. Statistical analyses were performed in R software version 4.4.1 (R Core Team, 2024), using the fat2.dic() function from the ExpDes.pt package (Ferreira, Cavalcanti & Nogueira, 2021).

2.3 Mycelial growth of *C. cubensis*

Mycelial growth of *C. cubensis* was evaluated using the same culture media and temperatures used for sporulation purpose. A mycelium plug of 5-mm diameter from a 7-day-old culture was transferred from the edge of the colony to Petri dishes (90×15 mm) containing 20 mL of culture medium. The plates were kept at 25 ± 1°C and 28 ± 1°C, under a 12-hour photoperiod. Colony diameter was measured with a caliper daily in two perpendicular directions until the mycelium reached the edge of one of the dishes. Mycelial growth was calculated as the average of the measurements. Data were transformed using the decimal logarithm (log_{10}), analysis of variance was performed, and means were compared using Tukey's test at a 5% significance level, conducted with R software (R Core Team, 2024). The linear mixed model was fitted using the lme() function from the nlme package (Pinheiro, Bates, & R Core Team, 2025).

2.4 Effect of light regime and incubation period on conidial germination of *C. cubensis*

A 30-µL droplet of conidial suspension, adjusted to a concentration of 10^5 conidia mL^{-1}, was deposited at the center of a Petri dish (60×15 mm) containing 10 mL of agar water (WA). The plates were kept at 28 ± 1°C under two light regimes: continuous light and continuous darkness. Conidial germination was halted by adding 10-µL lactofuchsin onto the suspension droplet after incubation periods of 12 and 24 hours. The percentage of germinated conidia was determined using a light microscope at ×100 magnification, by counting the conidia and considering those with a differentiated germ tube.

The experiment was carried out in a completely randomized design with a 2 (light regimes) × 2 (wetness periods) factorial scheme, with seven replicates. Each Petri dish was considered an experimental unit, with 100 conidia evaluated per replicate. The experiment was carried out twice. Data were transformed using $Log_{10}(X + 1)$ and subjected to analysis of variance with means compared by Tukey's test at a 5% significance level. Analyses were performed using R software version 4.4.1 (R Core Team, 2024), employing the lme4 (Bates et al., 2015) and lmerTest (Kuznetsova, Brockhoff & Christensen, 2017) packages.

2.5 Effect of temperature and incubation period on conidial germination of *C. cubensis*

To evaluate the effect of temperature and incubation period, an experiment similar to the previous one was conducted by depositing 30 µL of a suspension adjusted to 10^5 conidia·mL^{-1} onto Petri dishes (60×15 mm) containing WA. The plates were incubated at 25 ± 1°C and 28 ± 1°C under continuous light. Conidial germination was halted by adding 10-µL lactofuchsin onto the suspension droplet after incubation periods of 3, 6, 12, and 24 hours. The percentage of germinated conidia was determined by counting those exhibiting a differentiated germ tube, under a light microscope at ×100 magnification.

The experiment was conducted in a completely randomized design with a 2 (temperatures) × 4 (wetness periods) factorial arrangement, with five replicates. Each Petri dish was considered an experimental unit, with 100 conidia evaluated per replicate. The experiment was carried out twice. Data were transformed using Log_{10} and subjected to analysis of variance with means compared using Tukey's test at a 5% significance level. Analyses were performed using R software version 4.4.1 (R Core Team, 2024), employing the lme4 (Bates et al., 2015) and

lmerTest (Kuznetsova, Brockhoff & Christensen, 2017) packages for fitting and evaluating linear mixed-effects models.

2.6 Reproduction of wilting symptoms caused by *C. cubensis* in eucalypt

The experiment was carried out using six plants per treatment on 90-day-old rooted mini-cuttings of the *E. grandis* × *E. urophylla* hybrid (clone 200000837), which were kept in greenhouse for 75 days until they reached a stem diameters of 4 cm. The plants were grown in 2-L pots filled with a mixture of Tropstrato® and Floreiras e Vasos® substrates in a 1:1 ratio, supplemented with 500 g of superphosphate and 500 g of Osmocote® per 100 kilogram of substrate. The test was repeated once.

In an attempt to reproduce the wilting symptoms observed in eucalypt mini-stumps, the following methods of inoculation (treatments) of *C. cubensis* were tested: 1 - deposition of mycelial disk at 2.5 cm above the collar region over wounds made with a cork borer; 2 - wounding of branches with pruning shears followed by spray inoculation with a conidial suspension; 3 - wounding of branches with pruning shears previously immersed in a conidial suspension; 4 - wounding the stem base with a scalpel followed by spray inoculation with a conidial suspension; 5 - wounding of the stem base with a scalpel previously immersed in a conidial suspension; 6 - spray inoculation with a conidial suspension without wounding.

The *C. cubensis* conidial suspension used for spray inoculation (treatments 2, 4, and 6) was adjusted to 10^6 conidia·mL^{-1}, as previously described, and 10 mL of the suspension were applied towards each plant. In treatments 2 and 3, in which plants were pruned with pruning shears, three branches per plant were pruned immediately before inoculation. In treatment e, three superficial wounds were made 2.5 cm above the collar region using a scalpel. For the treatment 1, a bark disk was removed with a cork borer (ø = 5 cm) eight cm above the collar region and a mycelium disk of the same diameter, taken from the edge of a 7-day-old colony of the isolate, was placed onto the wound. To maintain moisture, the inoculated area was sealed with plastic film. For the control treatments, inoculations were performed using the same methods, but replacing the conidial suspension and mycelial disk with sterilized distilled water and PDA disk, respectively. Plants were conditioned in a mist irrigation chamber for 24 hours and subsequently maintained in a growth chamber (28 ± 2°C, 12-hour photoperiod). Plants were evaluated daily until the onset of symptom. Branches and stems were dissected to observe internal tissue necrosis at 30 days after inoculation (dai).

Disease incidence was expressed as the percentage of wilt plants in relation to the total number of inoculated plants. To fulfill Koch's postulates, the outer bark of stems and branches of symptomatic plants was aseptically removed with a sterile scalpel, and fragments of the inner necrotic tissue were transferred to PDA. The Petri dishes were then incubated at 28 °C.

3. RESULTS

3.1 Conidial sporulation of *C. cubensis*

There was a significant interaction between culture medium with eucalypt stem fragment and temperature ($p < 0.05$), indicating that the effect of temperature on the sporulation of conidia depends on the culture medium. The highest production of conidia was observed at 28 °C. At this temperature, there was no significant difference between PDA and PCA, both of which were superior to OMA. At 25 °C, the highest production of conidia was observed in PDA, followed by PCA and OMA (Table 1).

Table 1. Conidial sporulation of *C. cubensis* on PDA (potato dextrose agar), PCA (potato carrot agar), and OMA (oatmeal agar) culture media with eucalypt stem fragments at 25 °C and 28 °C.

Culture medium	Temperature	
	25°C	28°C
OMA	2.3×10^6 cB	5.2×10^6 bA
PCA	6.1×10^6 bB	8.9×10^6 aA
PDA	7.8×10^6 aB	9.9×10^6 aA

Averages followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically according to Tukey's test ($\alpha \leq 0.05$).

In the absence of eucalypt stem fragments, the interaction between culture media and temperature was significant according to analysis of variance ($p < 0.05$), indicating that the production of conidia by *C. cubensis* was influenced by those factors. Except for PDA, temperature of 25 °C resulted in higher production of conidia in BCA and OMA. At this temperature, the highest concentration of conidia was found in BCA, followed by PDA and OMA. At 28 °C, the highest production of conidia was on PDA, followed by PCA and OMA (Table 2).

Table 2. Conidial sporulation of *C. cubensis* on PDA (potato dextrose agar), PCA (potato carrot agar), and OMA (oatmeal agar) culture media without eucalypt stem fragments at 25 °C and 28 °C.

Culture medium	Temperature	
	25°C	28°C
OMA	5.5 x 10^5 bA	3.6 x 10^5 cB
PCA	5.9 x 10^6 aA	1.9 x 10^6 bB
PDA	6.3 x 10^5 bB	4.3 x 10^6 aA

Averages followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically according to Tukey's test ($\alpha \leq 0.05$).

The influence of eucalypt stem fragments can be observed in Figure 1, with higher sporulation when the isolate was cultivated on PDA with a fragment (Figure 1a), compared to when the mycelium developed without it (Figure 1b).

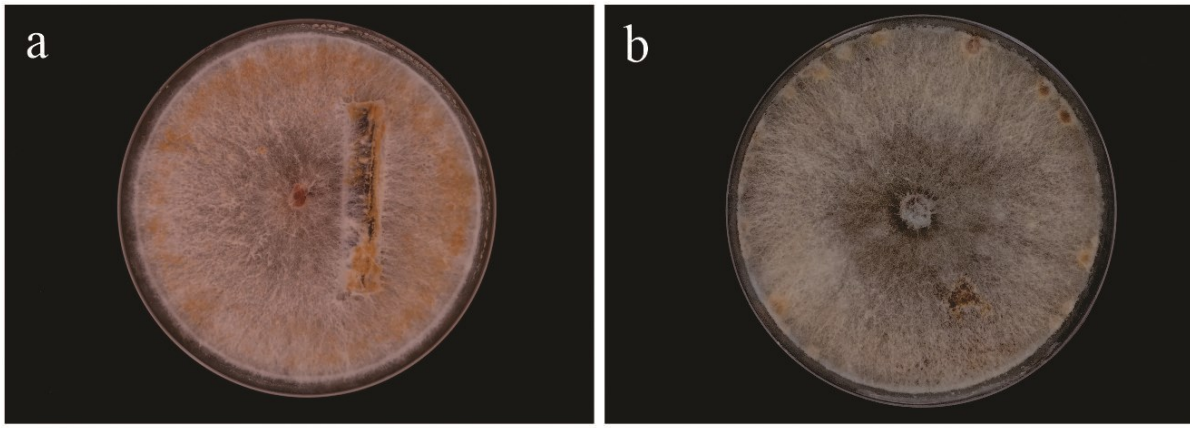

Figure 1. Aspect of the conidial **s**porulation of *Chrysoporthe cubensis* on potato dextrose agar (PDA) with (a) and without (b) eucalypt stem fragment, at 28°C after 13 days of incubation.

3.2 Mycelial growth of *C. cubensis*

Mycelial growth measurements were carried out over four days, when the *C. cubensis* colonies reached the edge of the PDA dish incubated at 28 °C. The interaction between culture media and temperature was significant at the 5% probability level ($p < 0.001$). Although the presented means are arithmetic, the statistical analysis was based on geometric means estimated from the transformed model. No significant difference of mycelial growth was observed on PDA at 25 °C (58 mm) and at 28 °C (67.1 mm). On the other hand, increasing the temperature from 25°C to 28°C promoted mycelial growth from 27 mm to 51 mm on PCA and from 17.2 mm to 28.9 mm on OMA. When comparing the average mycelial growth across the different media at each temperature, PDA provided the largest colony diameters, followed by PCA and OMA (Table 3).

Table 3. Mycelial growth (mm) of *Chrysoporthe cubensis* on PDA (potato dextrose agar), PCA (potato carrot agar), and OMA (oatmeal agar) at 25 °C and 28 °C.

Culture medium	Temperature	
	25°C	28°C
OMA	17.2 cB	28.9 cA
PCA	27.0 bB	51.0 bA
PDA	58.0 aA	67.1 aA

Averages followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically according to Tukey's test ($\alpha \leq 0.05$).

3.3 Effect of light regime and incubation period on conidial germination of *C. cubensis*

The interaction between light regime and wetness period was significant at the 5% probability level, according to the analysis of variance. No significant difference was observed between incubation period in the light (32% at 12-hour incubation and 30.4% at 24-hour incubation). On the other hand, in the dark, a higher percentage of conidial germination was obtained after 24 hours of incubation (increasing from 17.1% at 12-hour incubation to 35.1% at 24-hour incubation). The interaction between light regime and incubation period also affected conidial germination. After 12 hours of incubation, higher germination occurred in the light. However, after 24 hours, no statistical difference was observed for germination in the light and in the dark (Table 4).

Table 4. Germination of conidia of *Chrysoporthe cubensis* influenced by light regime (continuous light and dark) and incubation periods (12 and 24 hours).

Light regime	Incubation period	
	12 hours	24 hours
Dark	17.1 bB	35.1 aA
Light	32.0 aA	30.4 aA

Averages followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically according to Tukey's test ($\alpha \leq 0.05$).

3.4 Effect of temperature and incubation period on conidial germination of *C. cubensis*

The interaction between temperature and incubation period was significant at the 5% probability level according to the analysis of variance. The *C. cubensis* conidia did not germinate under three and six hours of wetness. A significant increase in the conidia germination was observed with the extension of the incubation period at both temperatures. At 25 °C, the germination rate increased approximately 11.0 times from the 12-hours (1.25%) to 24-hours (13.1%) of incubation, while at 28 °C, it doubled, rising rose 16.2% to 33.9% over the same incubation periods (Table 5).

Table 5. Germination of *Chrysoporthe cubensis* influenced by incubation period (12 and 24 hours) and temperature (25 °C and 28 °C).

Temperature (°C)	Incubation period	
	12 hours	24 hours
25	1.2 bB	13.1 bA
28	16.2 aB	33.9 aA

Averages followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically according to Tukey's test ($\alpha \leq 0.05$).

3.5 Reproduction of wilting symptoms caused by *C. cubensis* in eucalypt

The onset of wilting was observed at 13 and 17 dai in the first and second experiments, respectively, occurring exclusively in plants pruned with shears previously immersed in a conidial suspension of *C. cubensis* (Figure 2B), and in plants pruned and subsequently inoculated by spraying with the conidial suspension (Figure 2C).

By 23 dai, all plants in treatments 2 and 3 showed 100% wilting incidence. Plants were dissected under a stereomicroscope and the symptomatic ones presented necrotic lesions in the internal tissues of the branches, with average lengths of 30.4 mm and 25.4 mm in treatments 2 and 3, respectively. Plants inoculated with mycelial plugs also exhibited necrotic lesions in the internal stem tissues, with an average length of 43.2 mm. The pathogen was successfully re-isolated from the internal tissues of symptomatic plants, confirming its pathogenicity.

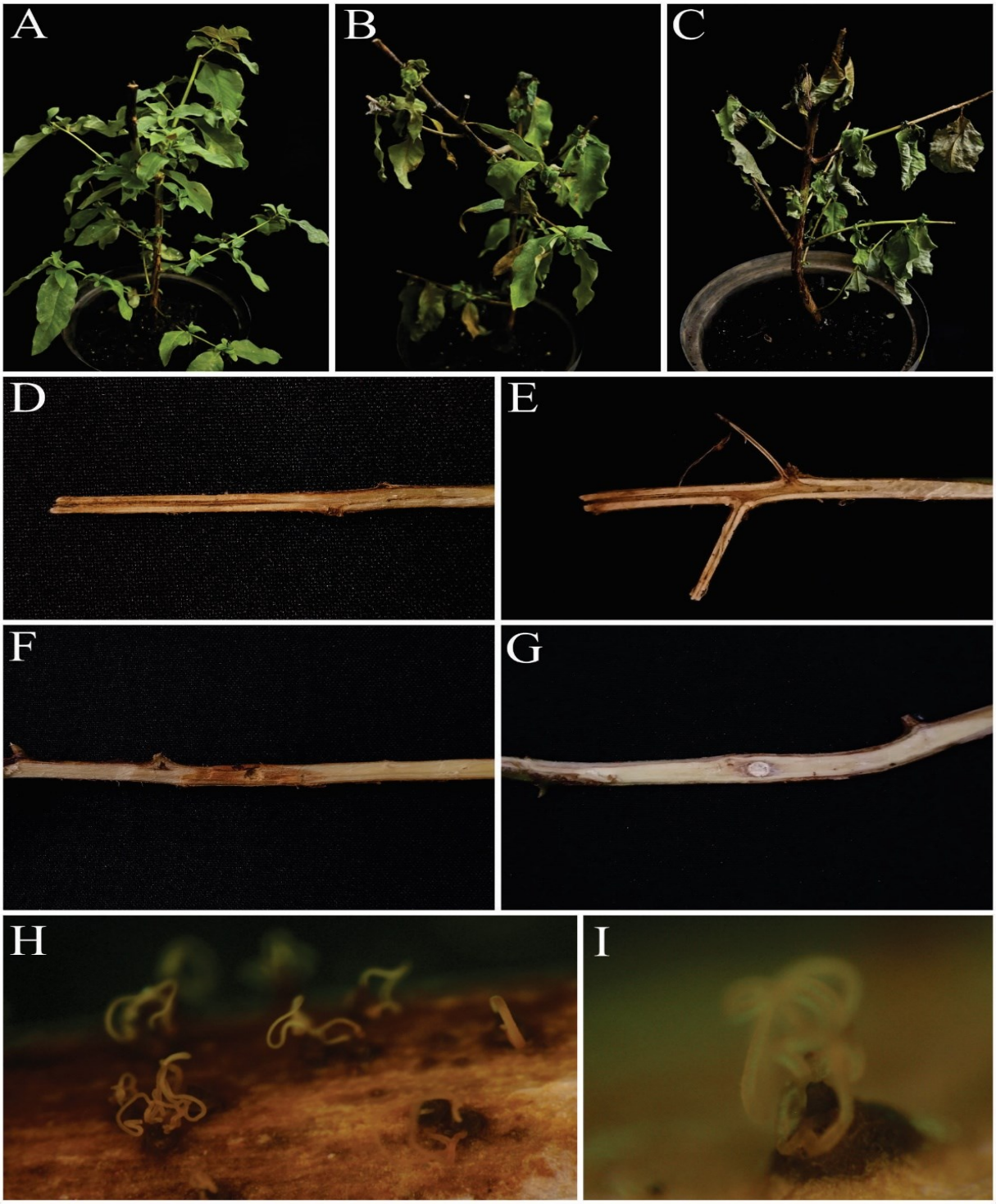

Figure 2. Reproduction of wilting symptoms caused by *Chrysoporthe cubensis* into approximately 6-month eucalypt rooted mini-cutting. (A) Plant pruned and sprayed with sterilized water; (B) Plant pruned and inoculated with pruning shear previously immersed in a conidial suspension; (C) Plant pruned and inoculated by spraying conidial suspension; (D) Necrotic lesion in tissue of plant inoculated with pruning shear immersed in conidial suspension of *C. cubensis*; (E) Necrotic lesion in tissue of plant inoculated by spraying conidial suspension; (F) Plant inoculated by a mycelial disk; (G) Plant inoculated with a BDA disk; (H) Pycnidia of the pathogen forming cirrhi on a surface of a branch of plants sprayed with a conidia suspension; (I) Close-up of conidia forming cirrhu.

4. DISCUSSION

Chrysoporthe cubensis is geographically distributed in tropical and subtropical regions between latitudes 30° North and South, predominantly occurring in areas with high temperatures and rainfall (Hodges et al., 1979). In general, fungal growth and reproduction are influenced by several factors, such as temperature, light, and nutrient availability (Colhoun, 1973). In this study was demonstrated that conidial sporulation of *C. cubensis* was affected by the tested temperatures, culture medium and whether a eucalypt stem fragment was present on its surface. All tested culture media were suitable for promoting fungal sporulation. PDA, particularly at elevated temperatures, also supported high spore production in *Ceratocystis fimbriata*, *Apoharknessia eucalyptorum*, *Neoscytalidium dimidiatu*m, *Fusarium solani*, and *Neoscytalidium hyalinum* (Dutra et al., 2025; Brito et al., 2021; Garrett et al., 2019; Hohenfeld et al., 2018). PCA was also found to be suitable for the sporulation of *C. cubensis*. This medium has likewise been effective for the sporulation of *Choanephora* spp. (Oliveira and Bonaldo, 2022).

The temperature of 28 °C was found to be optimal for mycelial growth in all tested culture media and also favored the germination of *C. cubensis* conidia. According to Ferreira (1989), the optimal temperature range for mycelial growth of this species is between 27 and 30 °C. The effect of temperature was also demonstrated on the development of Chrysoporthe wilt, at 28°C was observed longer necrotic lesions on the stem of eucalyptus, when compared to 25°C (Martins et al. 2023). The growth rate of several isolates of *Endothia* (*Cryphonectria*) *parasitica*, isolated from American chestnut trees, varied little between 21 °C and 32 °C, although the fastest growth occurred between 27 °C and 32 °C (Anagnostakis and Aylor, 1984).

Luminosity was not a limiting factor for the *in vitro* germination of *Chrysoporthe cubensis* conidia. For *Botrytis cinerea* on eucalypt leaves, conidia were observed to germinate under both light and dark conditions, although the germination rate and grey mold intensity were higher in the absence of light (Caires et al., 2015). Likewise, the germination and infection of *Austropuccinia psidii* were negatively affected by the presence of light (Ruiz et al., 1989).

Conidial germination of *C. cubensis* increased with longer incubation periods at both 25 and 28 °C. This species shows higher conidial germination under prolonged moisture conditions, with germ tube emergence beginning after 11 hours of incubation (Bruner, 1917). In both incubation periods, 28 °C promoted higher germination rates, similarly to *Calosphaeria pulchella*, which also showed greater germination under higher temperatures (Li, Travadon & Trouillas, 2023).

Regarding the reproduction of wilting symptoms, this is the first time that eucalypt plants inoculated with *C. cubensis* have reproduced typical symptoms of this disease, which had been reported in mini-clonal hedge in Brazil. Therefore, up to now, Koch's postulates for *C. cubensis* isolates recovered from eucalypt mini-stumps with wilting symptoms have been only partially fulfilled by Martins et al. (2023). The inoculation procedure used by these authors in both studies was the same as that commonly employed to inoculate *C. cubensis* in eucalypt trees to reproduce canker symptoms in the field (Guimarães, 2010). A culture medium disk colonized by the fungus was taken from the plates using a cork borer and inserted just beneath the bark. However, this procedure only reproduced the necrotic lesions observed in the inner stem of symptomatic mini-stumps. The wilting symptoms were not observed.

These findings are important as they pave the way for further studies aimed at establishing control measures for Chrysoporthe wilt in eucalypt mini-stumps. Moreover, they provide insights into the potential role of pruning shears in the dispersal of the pathogen and/or in facilitating the entry of *C. cubensis* into mini-stumps through pruning wounds. These results are consistent with the fact that pathogens typically require injuries to infect eucalypt trees and cause canker under field conditions, often penetrating through wounds such as bark cracks, branch–trunk junctions, or other forms of physical damage (Ferreira, 1989). To gain a deeper understanding of the pathogen dispersion mechanism, future studies should be conducted using mini-stumps in mini-clonal hedge conditions to confirm these findings and clarify the role of pruning shears in the dispersion of *C. cubensis*. Additional assays will be conducted to better understand the role of wounds in pathogen penetration, as well as to investigate the movement of the fungus within plant tissues after penetration in order to elucidate its infection process.

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