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Morphophysiological and molecular characterization of *Cercospora* spp. in *Fragaria* × *ananassa* Duch.

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ABSTRACT: The strawberry crop (*Fragaria* × *ananassa* Duch.) originated in Europe and has a wide geographical distribution. In Brazil, cultivation is predominant in the southern regions of the country. Foliar diseases are one of the main problems of the crop and are caused by fungal species of different genera. Recently, *Cercospora* spp. has been reported as a causal agent of cercosporiosis on strawberry leaves. Thus, the objective of this study was to evaluate the pathogenicity and to morphophysiologically and molecularly characterize isolates of *Cercospora* spp. obtained from strawberry leaves. Pathogenicity was evaluated on detached leaves, morphophysiological characterization on different culture media, and molecular identification by the actin and elongation factor1-alpha regions. The morphological characteristics were similar to those found in species of the genus *Cercospora*. Cercosporiosis in strawberry is caused by a complex of species of the genus *Cercospora*, among them *C. kikuchii*.

Key words: actin; cercosporiosis; elongation factor; strawberry

Caracterização morfofisiológica e molecular de *Cercospora* spp. em *Fragaria* x *ananassa* Duch.

RESUMO: A cultura do morangueiro (*Fragaria* × *ananassa* Duch.) é originaria da Europa e apresenta ampla distribuição geográfica. No Brasil, o cultivo é predominante nas regiões sul do país. As doenças foliares são um dos principais problemas da cultura e são causadas por espécies de fungos de diferentes gêneros. Recentemente, *Cercospora* spp. foi relatado como agente causal de cercosporiose em folhas de morangueiro. Dessa forma, o objetivo deste estudo foi avaliar a patogenicidade e caracterizar morfofisiológica e molecularmente isolados de *Cercospora* spp. obtidos de folhas de morangueiro. A patogenicidade foi avaliada em folhas destacadas, a caracterização morfofisiológica em diferentes meios de culturas e a identificação molecular pelas regiões actina e fator de elongação1-alfa. As características morfológicas foram semelhantes às encontradas em espécies do gênero *Cercospora*. A cercosporiose em morangueiro é causada por um complexo de espécies do gênero *Cercospora*, entre essas *C. kikuchii*.

Palavras-chave: actina; cercosporiose; fator de elongação; morangueiro

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Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is cultivated on all continents. The area planted to this fruit worldwide is estimated to be 384,668 hectares (ha) (FAOSTAT, 2020). Brazil has favorable climatic conditions for the development of strawberry because it has subtropical climate regions, which provides a good adaptation of the different cultivars available on the market (Rosa et al., 2013).

In Brazil, the area planted with strawberry is approximately 5,200 ha, with a production of over 200,000 tons (Antunes et al., 2021). This production is predominantly in small family-based areas, with the participation of available labor supplemented by occasional contracts in periods of greater demand (Madail, 2016).

The productivity and quality of strawberries are influenced by different factors. These can be of abiotic or biotic origin, and among the biotic factors are the pathogenic fungi, causal agents of different diseases that affect the strawberry crop. Foliar diseases are one of the main problems of the crop and are caused by fungal species of different genera. Recently, Krahn et al. (2022) reported the occurrence of a new leaf spot in the strawberry crop, called cercosporiosis, caused by the fungus *Cercospora* cf. *malloti*.

The symptoms of cercosporiosis in strawberry are observed on the leaves during the entire cycle of the crop, especially during the reproductive period. The leaves present circular spots with an initial reddish-purple to dark brown coloration, which later increase in size, presenting the center of the lesion with a whitish coloration (Krahn et al., 2022).

Thus, the objective of this study was to evaluate the pathogenicity and to morphophysiologically and molecularly characterize isolates of *Cercospora* spp. obtained from strawberry leaves.

Materials and Methods

Obtaining isolates of *Cercospora* spp.

Leaves with characteristic symptoms of cercosporiosis were collected between the months of April and October 2019, in strawberry producing properties in several municipalities of Rio Grande do Sul state, Brazil, and in the municipality of São João do Oeste, Santa Catarina state, Brazil.

The collected leaves were stored in plastic bags under refrigeration and sent to the Laboratório de Fitopatologia Elocy Minussi, at the Universidade Federal de Santa Maria, to perform the identification and isolation of the pathogen. Thus, direct isolation was performed by transferring part of the fungal structures from the leaf lesion to Petri plates containing PDA culture medium (potato - dextrose - agar) and 0.05 mg of streptomycin 100mL^{-1} of medium, and then the plates were placed in an incubator with a 12 hour photoperiod and temperature of 25 ± 2 °C for 15 days. After identification and isolation of the pathogen, monospore cultures were performed to obtain purified isolates (Fernandes, 1993). From the collections 13 isolates were obtained, as shown in Table 1.

Table 1. Isolates of *Cercospora* spp. from strawberry (*Fragaria* × *ananassa* Duch.) crops in different municipalities of Rio Grande do Sul and Santa Catarina states, Brazil.

Isolated	Municipality	Coordinates
M1	Santiago – RS	29°10'59.1" S, 54°50'59.9" W
M2	Ijuí – RS	28°22'06.6" S, 53°58'30.0" W
M3	Capão do Cipó – RS	29°02'08.7" S, 54°38'06.4" W
M4	Santa Maria – RS	29°43'19.6" S, 53°43'04.6" W
M5	Coronel Barros – RS	28°23'10.4" S, 54°04'15.1" W
M6	Santa Maria – RS	29°43'19.6" S, 53°43'04.6" W
M7	São João do Oeste – SC	27°05'49.6" S, 53°35'25.9" W
M8	Santa Maria – RS	29°38'57.2" S, 53°55'04.5" W
M9	Agudo – RS	29°38'33.7" S, 53°15'10.1" W
M10	Passo Fundo - RS	28°13'48.5" S, 52°22'45.2" W
M11	Santa Maria – RS	29°38'57.2" S, 53°55'04.5" W
M12	São João do Polêsine – RS	29°40'30.2" S, 53°31'16.0" W
M13	Paraíso do Sul – RS	29°45'01.3" S, 53°03'42.1" W

Morphophysiological characterization

To evaluate morphological characteristics such as colony coloration, size and number of conidial septa, and the final colony diameter (FCD) and sporulation of all isolates, PDA medium discs with 6 mm diameter of *Cercospora* spp. isolates, obtained from colonies with 15 days of growth, were transferred to other plates with PDA medium and kept at 25 ± 2 °C, with 12 hours of photoperiod, in a climate-controlled incubator, for a period of 15 days. For this, five repetitions were used, each consisting of one plate. The mycelial growth of each isolate was evaluated at 15 days of incubation by measuring the average colony diameter in two diametrically opposite directions. Next, colony staining was determined by observation and comparison of the top surface of the Petri dishes, aided by the Munsell color chart (Munsell Color, 2009).

The sporulation was evaluated at 20 days of incubation, 15 days with temperature at 25 ± 2 °C and photoperiod of 12 hours, and the other days with continuous ultraviolet light. For each isolate, the length and width of 10 conidia, as well as the number of septa were measured with the aid of an optical microscope, with micrometer attached, at 40× magnification.

For the evaluation of physiological characteristics, such as mycelial growth and sporulation, two pathogen isolates were grown on different media. Discs of PDA medium, 6 mm in diameter containing mycelium of *Cercospora* spp. obtained from colonies with 15 days of growth were transferred to plates containing the malt culture medium (malt extract, agar and distilled water), carrot medium (CAR: carrot extract, agar and distilled water) and vegetable juice medium (V8: commercial V8 juice, $CaCO_3$, agar and distilled water). The plates with the isolates were kept at 25 ± 2 °C, with 12 hours of photoperiod, in a climate-controlled incubator, for a period of 15 days for the evaluation of FCD.

The sporulation was evaluated at 20 days of incubation, 15 days with temperature at 25 ± 2 °C and photoperiod of 12 hours, and the other days with continuous ultraviolet light. On each plate, 10 mL of sterile distilled water was added, plus one drop of the emulsifier Tween 80®, after which the colonies were scraped off and sieved through a double layer

of gauze. The suspension was stirred for 30 seconds and, with the help of a micropipette, 1 mL was removed and spread in the Neubauer chamber for the subsequent estimation of conidia concentration (conidia mL^{-1}). Data were subjected to analysis of variance and means were compared by Scott-Knott test using SISVAR 5.3 software (Ferreira, 2019).

Molecular characterization

DNA extraction and sequencing of the isolates was performed at the Instituto Biológico de São Paulo, according to the method described by Doyle & Doyle (1987) from mycelium grown in culture medium. The extracted genomic DNA sample was submitted to polymerase chain reaction (PCR) for amplification of the internal transcribed spacer (ITS) region of the rDNA and segments of calmodulin, elongation factor 1-alpha, and actin genes. The primer oligonucleotides for the ITS region were SR6R and LR1 (Muniz et al., 2010), for the calmodulin gene were CAL-228F and CAL-737R, for the elongation factor gene were EF1-728F and EF1-986R, and for the actin gene were ACT-512F and ACT-783R (Carbone & Kohn, 1999). The sequenced fragments were analyzed using BioEdit software (Hall, 1999). The nucleotide sequences obtained were compared with those already existing for *Cercospora* spp. in GenBank using the Basic Local Alignment Search Tool (BLAST). The sequences from GenBank that presented the highest scores were selected and aligned with those from the isolates, by the ClustalW algorithm, in addition, the phylogenetic analysis was conducted by adopting the Maximum Likelihood method, by the Kimura 2-parameter model, with 1,000 replicates by MEGA version 10 software (Kumar et al., 2018).

Pathogenicity test

For the pathogenicity test, detached leaves of strawberry cv. Camarosa and six isolates of *Cercospora* spp. were used (Rosa & Menezes, 2001). The asepsis of the leaves was done by immersion in a 70% alcohol solution for 30 seconds, followed by immersion in 0.5% sodium hypochlorite for 30 seconds, and finally rinsed in sterilized distilled water for another 30 seconds and dried in a laminar flow chamber. After asepsis, the leaves were placed in "gerbox" boxes, with two sheets of "germitest" paper moistened with sterilized distilled water.

The experimental design used was entirely randomized, with three repetitions consisting of two leaves. Each leaf received nine discs of PDA medium containing the mycelium of the fungus from 20-day-old colonies. Wounds were made on the leaves using a histological needle. After inoculations, the boxes were closed and stored in a climate controlled chamber at 25 ± 2 °C and a 12 hour photoperiod for 14 days. The evaluation took place on the fourteenth day post-inoculation. The inoculated site was measured orthogonally to obtain the diameter of the lesions (mm) and considered infected when the necrotic spots were significantly larger than the control. The leaves of the control treatment were wounded with a histological needle and PDA discs without the pathogen were added. The incidence of infection was calculated by the formula [Incidence (%) = (infected sites / inoculated sites) × 100] (Poletto, 2022).

Results and Discussion

Morphological characterization

The 13 isolates of *Cercospora* spp. were characterized for daily mycelial growth and then the final colony diameter (FCD) and daily mycelial growth rate (MGR) were determined, as well as sporulation and conidia size. The isolates were registered in the Sisgen by the number: A57AE19.

Table 2 shows the means of the analyzed variables and, by the mean comparison test, it can be seen that there was a statistical difference between the isolates. Statistical analysis allowed the isolates to be grouped into four groups for the FCD and MGR variables, while for sporulation, three.

Table 2. Morphological characters of *Cercospora* spp. isolates on PDA culture medium.

Isolated	FCD (mm)	MGR (mm day^{-1})	Sporulation (conidia mL^{-1})	Conidia		
				Width (µm) (Interval)	Length (µm) (Interval)	Septo (Interval)
M1	53.5 a*	3.56 a	7.0×10^4 b	9.2 (8-10)	43.4 (40-45)	7 (6-8)
M2	51.11 b	3.40 b	8.8×10^4 b	8 (7-8)	39.2 (38-40)	5.4 (5-6)
M3	48.37 b	3.22 b	1.2×10^5 a	8.2 (8-9)	37.3 (36-39)	5.8 (5-7)
M4	44.62 c	2.97 c	1.9×10^5 a	3.1 (3-4)	7.3 (5-8)	3.6 (3-4)
M5	42.45 c	2.83 c	1.5×10^5 a	3.7 (3-4)	8.5 (8-10)	4 (4-4)
M6	36.41 d	2.44 d	2.1×10^4 c	4.1 (3-4)	10.7 (9-12)	3.6 (3-4)
M7	52 .44 a	3.49 a	7.3×10^4 b	10.5 (9 -12)	52.6 (50-56)	6.8 (7-8)
M8	47.67 b	3.17 b	9.4×10^4 b	3.3 (3-4)	6.8 (5-8)	2.8 (2-3)
M9	47.63 b	3.17 b	7.8×10^4 b	3 (2-4)	10.2 (9-10)	3.2 (3-4)
M10	49.39 b	3.29 b	1.4×10^5 a	10.7 (10-12)	74.4 (70-77)	11 (10-11)
M11	56.44 a	3.76 a	1.4×10^5 a	13.3 (13-14)	84.4 (80-87)	16.6 (14-18)
M12	49.96 b	3.33 b	1.4×10^5 a	9.8 (9-10)	80.6 (75-86)	15.8 (14-17)
M13	47.58 b	3.17 b	1.3×10^5 a	9.8 (8-11)	69.4 (68-70)	14.2 (13-16)
CV (%)	9.50	9.50	20.16			

* Averages followed by the same letter in the column do not differ statistically by the Scott-Knott test ($p \geq 0.05\%$); CV - coefficient of variation; FCD - final colony diameter; MGR - mycelial growth rate.

The group with the highest mean FCD scores was composed of *Cercospora* isolates M1, M7, and M11, and the group with the lowest average was M6 alone. Consequently, the TCM was also proportional to these isolates, with values of 3.56, 3.49, and 3.76 mm day^{-1}, for M1, M7, and M11, respectively, and the lowest was 2.44 mm day^{-1} for isolate M6 (Table 2).

The isolates M3, M4, M5, M10, M11, M12, and M13 formed the group with the highest sporulation, in which, the isolate M4 stood out (1.9×10^5 conidia mL^{-1}). The isolate M6 alone showed the lowest sporulation: 2.1×10^4 conidia mL^{-1}, while the other isolates showed intermediate sporulation. Also in Table 2, the variables conidia width, length, and number of septa showed variation among the isolates. In addition to these evaluated characteristics, the presence of conidiophores was observed (Figure 1A).

In general, the isolates had slow growth with formation of aerial mycelium, pinkish in color, and, as the colony aged, they showed grayish color. Li et al. (2021), also observed isolates of *Cercospora* cf. *citrulina* with slow growth, however, the mycelium coloration was white to gray. Although, it is a characteristic of the genus *Cercospora*, Pittner et al. (2016), studying the development of *Cercospora beticola* reported a more accelerated mycelial growth of the colony, as in seven days the final colony diameter ranged from 30.75 to 46.50 mm. The differences in morphological and physiological characters found in the present study may be related to the conditions of the culture medium, as well as related to the natural variability of populations of the genus *Cercospora*.

The conidia were hyaline, monoseptate to multiseptate with various shapes, ranging from filiform, straight to slightly curved. The average size of the conidia was distinct among the isolates, as was the number of septa. For the isolate M6 it was 4.1 × 10.7 µm and an average of 3.6 septa. M11 in turn showed larger conidia, 13.3 × 84.4 µm and an average number of 16.6 septa. These characteristics are consistent with fungi of the genus *Cercospora*. Andrade (2016), reports that he found a wide variation in the size of *Cercospora* spp. conidia on cucurbits, with width measurements 4 to 8 µm and length ranging from 40 to 216 µm. The number of septa found in the author study ranged from 3 to 20 and varied in shape. Similar results were also found by Queiroz et al. (2020), in conidia of *C. apii* that were 2.5 to 4 µm width and 92 to 210 µm length.

After morphological characterization, the data related to mycelial growth (FCD, MGR) and sporulation (conidia size: width and length, number of septa, and sporulation) were submitted to the UPGMA (Unweighted Pair Group Method with Arithmetic Average) grouping method, and through the generated dendrogram, the isolates were grouped into three groups by the similarity between the averages of the characters, based on these, molecular identification and the pathogenicity test of two isolates per group were performed (Table 3).

Table 3. Groups of *Cercospora* spp. isolates obtained from strawberry leaves, based on the UPGMA (Unweighted Pair Group Method with Arithmetic Average) grouping method.

Group	Isolated
1	M10, M13*, M11*, and M12
2	M6*, M4, M5, M8*, and M9
3	M7*, M1, M2*, and M3

* Isolates used for pathogenicity testing and molecularly identified.

Pathogenicity test

Isolates M2, M6, M7, M8, M11, and M13 of Cercospora spp. were pathogenic to strawberry (*Fragaria* × *ananassa* Duch.) leaves. The first symptoms observed were small circular spots, initially light brown in color with a yellowish halo (Figure 2C). At the end of the test, the leaves had brown to purple spots and cream to whitish-colored lesion centers (Figure 2D), with an average lesion diameter ranging from 2.52 to 3.08 mm among isolates. The incidence of the disease was 100%, since all inoculated sites had a lesion. Leaves that were inoculated with culture medium alone showed no symptoms.

Chai et al. (2021) observed similar symptoms on leaves of *Abelmoschus esculentus* when infected by *Cercospora* cf.

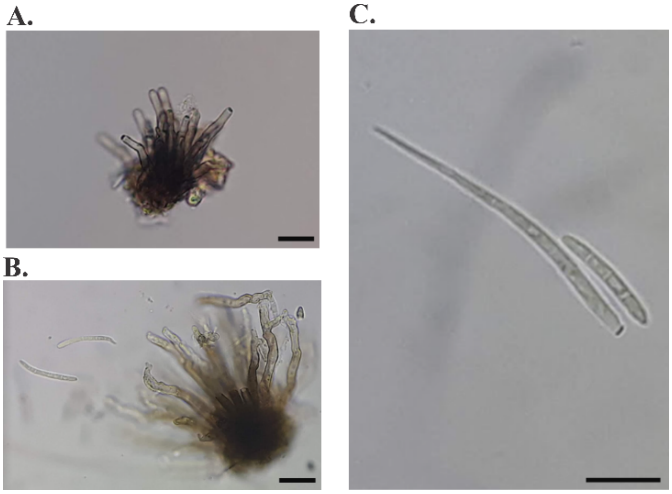


Figure 1. Conidiophores of *Cercospora* spp. on PDA medium (A), conidia and conidiophores (B), and different conidium sizes (C). Bar: 10 µm.

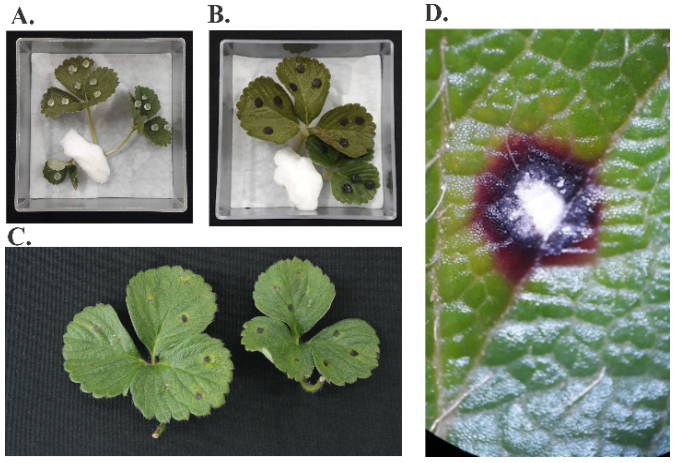


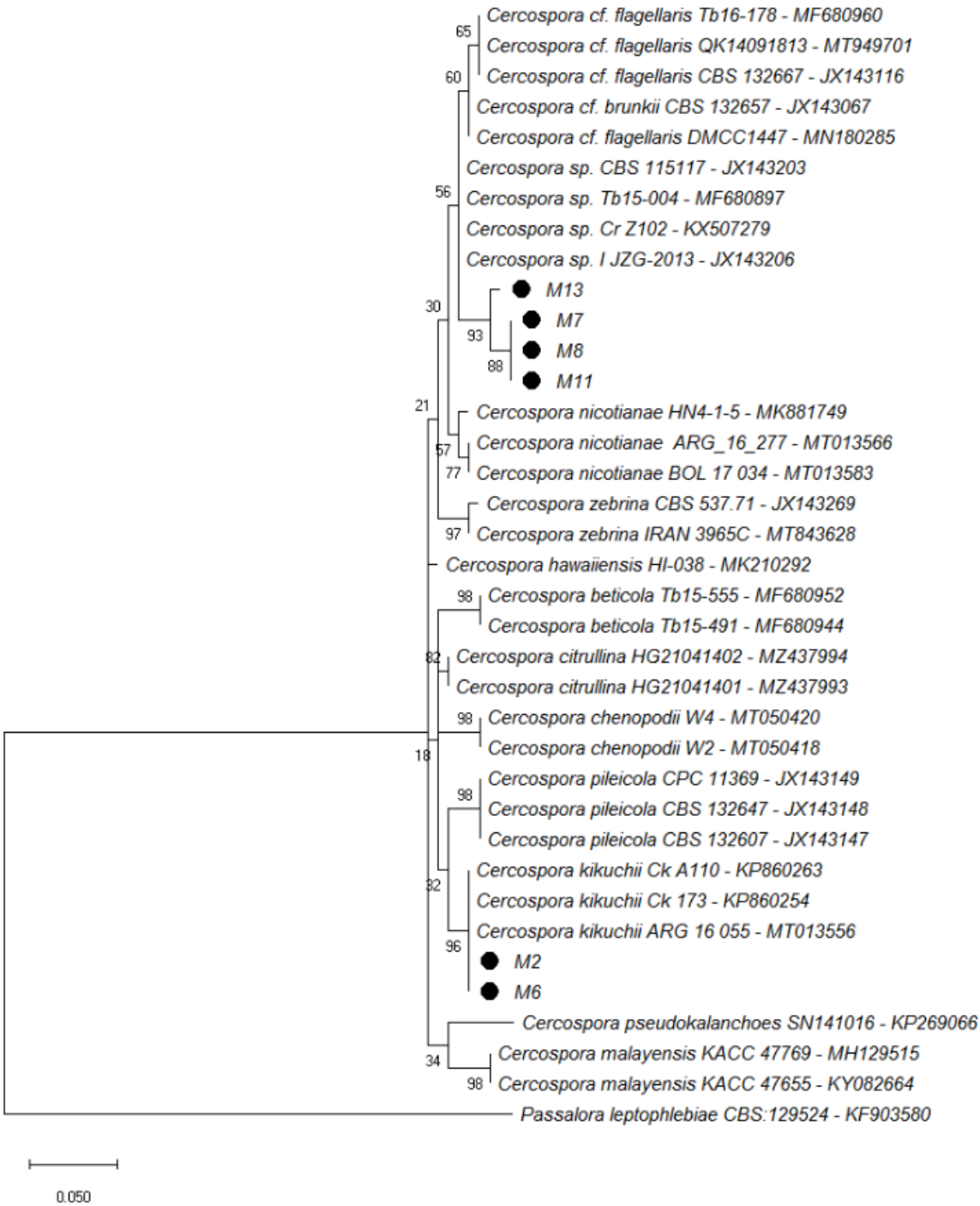
Figure 2. Witness with discs of culture medium without the pathogen (A), isolate M6 on strawberry leaves (B), lesions after 15 days of incubation with isolate M6 (C), and lesion caused by *Cercospora* spp. (D).

flagerallis. These symptoms are characteristic of the genus *Cercospora*, due to the ability of some species to produce a metabolite called cercosporin, which is toxic to plants and responsible for the purple coloration of leaves (Santos, 2015).

Molecular characterization

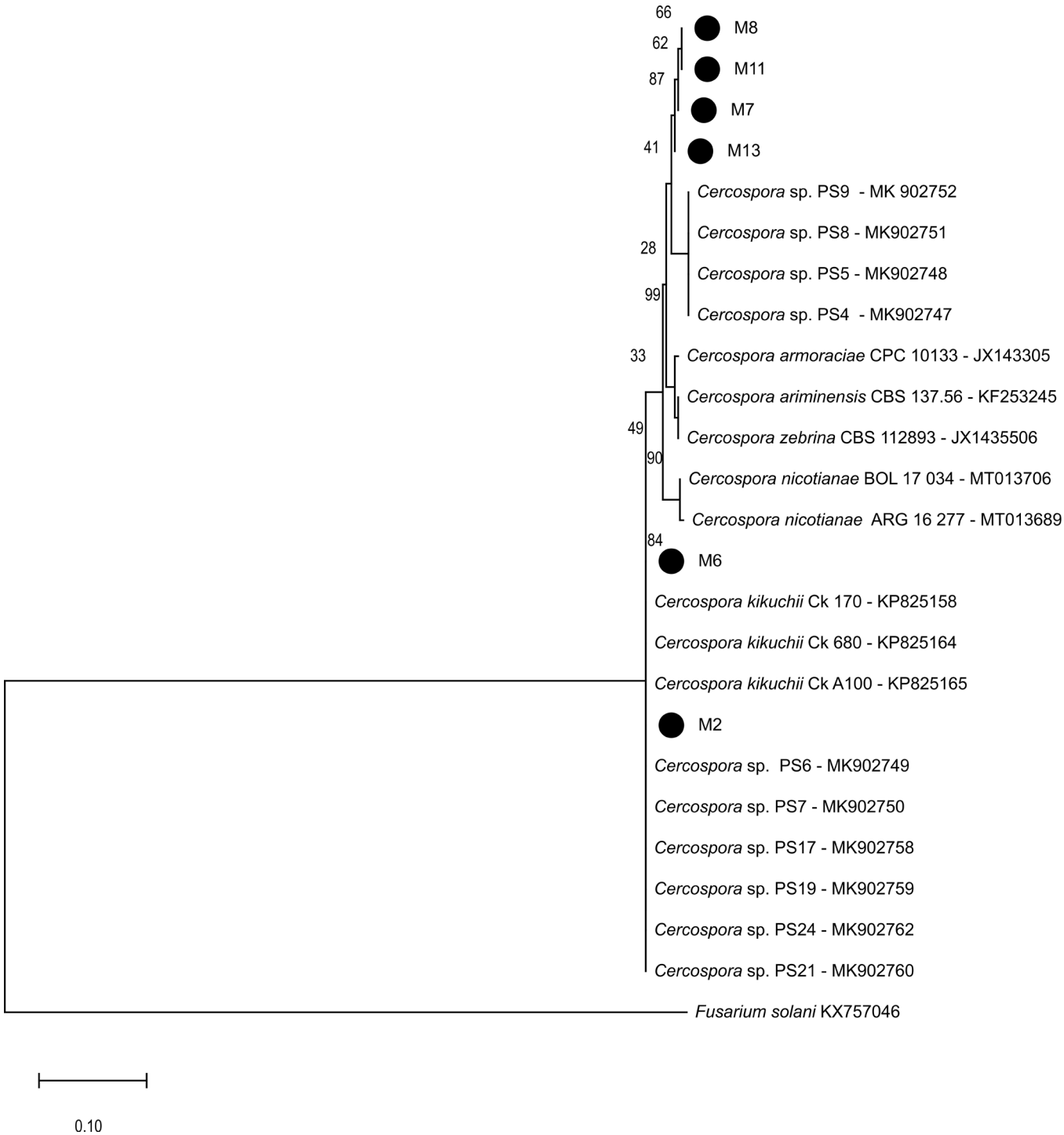
The ITS (internal transcribed spacer) regions of rDNA and the partial calmodulin gene (CAL) were inconclusive for all isolates and therefore will not be presented. Figures 3 and 4 represent the phylogenetic trees obtained by aligning isolates M2, M6, M7, M8, M11, and M13 with the actin (ACT) region (Figure 3) and elongation factor 1-alpha (Tef-1) (Figure 4) sequences of *Cercospora* isolates from the GenBank database.

Only isolates M2 and M6 were identified as *C. kikuchii*, as they were allocated in a distinct clade with *C. kikuchii* isolates with bootstrap support value of 96% for ACT region and 84% for Tef-1. For the ACT and Tef-1 region, isolates M7, M8, M11,



The numbers on the branches indicate the percentage of bootstrap analysis replicates in which the replicates were observed (1,000 replicates).

Figure 3. Phylogenetic tree obtained from the actin gene sequence (ACT) of isolates of *Cercospora* spp. associated with strawberry (*Fragaria* × *ananassa* Duch.) leaves, showing the phylogenetic relationships among species according to the Maximum Likelihood statistical method, with the Kimura 2-parameter model.



The numbers on the branches indicate the percentage of bootstrap analysis replicates in which the replicates were observed (1000 replicates).

Figure 4. Phylogenetic tree obtained from the sequence of the elongation factor 1-alpha gene of isolates of *Cercospora* spp. associated with strawberry (*Fragaria* × *ananassa* Duch.) leaves, showing the phylogenetic relationships among species according to the Maximum Likelihood statistical method, with the Kimura 2-parameter model.

and M13 were grouped into clades with species sequences of *C.* cf. *flagerallis*, *C.* cf. *brunkii* and *C. armoraciae*, *C. zebrina*, *C. nicotianae*, and *Cercospora* sp. species identification was inconclusive. However, it can be said that they belong to the genus *Cercospora*.

C. kikuchii is known as the causal agent of *Cercospora* leaf spot on leaves and purple spot on soybean (*Glycine max*) seeds. Recent studies have confirmed that these diseases are caused by a complex of different *Cercospora* species, including: *C. kikuchii*, *C.* cf. *flagellaris*, *C.* cf. *sigesbeckiae*, and *C. nicotianae* (Soares et al., 2015; Albu et al., 2016). Therefore, isolates with inconclusive identification may form a complex of species that cause cercosporiosis on strawberry.

Table 4 shows the accession codes of *Cercospora* spp. isolates deposited in Genbank for the actin and elongation factor 1-alpha regions.

Table 4. Genbank accession codes of *Cercospora* spp. isolates for the actin (ACT) and elongation factor 1-alpha (Tef-1) regions.

Isolated	Species	Region	
		ACT	Tef-1
M2	*C. kikuchii*	ON950243	ON975037
M6		ON950731	ON989006
M7	*Cercospora* sp.	ON960074	ON994187
M8		ON972466	ON995107
M11		ON960047	OP038545
M13		ON972438	OP004917

Morphophysiological characterization

For morphophysiological characterization on different culture media, isolates M6 and M11 were selected, due to their distinct characteristics, such as FCD, sporulation, and conidia size on PDA medium.

On the different culture media tested, the isolates showed distinct behaviors regarding FCD, sporulation, and the production of cercosporin. However, the V8 juice medium provided the best development conditions for M6 and M11, getting a FCD of 47.39 and 56.03 mm, respectively. As for sporulation it was 1.12×10^5 conidia mL^{-1} for M6 and 5.06×10^4 conidia mL^{-1} for M1 (Table 5).

Production of cercosporin was observed in both isolates of *Cercospora* spp. with the V8 medium providing the highest production of the toxin (Figure 5). This may be related to the composition of the medium, which is rich in micronutrients, and also to the ability of each isolate to metabolize the toxin, since not all *Cercospora* species produce the toxin (Świderska et al., 2020).

The V8 juice medium is a rich carbohydrate medium, because its composition includes different types of vegetables rich in sugars. Thus, it is considered a medium that provides good mycelial growth and sporulation of different species of the genus *Cercospora* (Hanada et al., 2002; Beckman & Payne, 1983). Ramírez et al. (2019) in a recent study, evaluating the sporulation of cercosporoid fungi with different culture media, reported that media composed with V8 juice provided the best conditions for sporulation of isolates of *Cercospora* sp. and *Pseudocercospora fumosa*, corroborating with the results obtained in the present study. Therefore, the culture medium with vegetable juice can be used for the development of new studies involving *Cercospora* isolates, as it provided the best conditions for growth, sporulation, and cercosporin production.

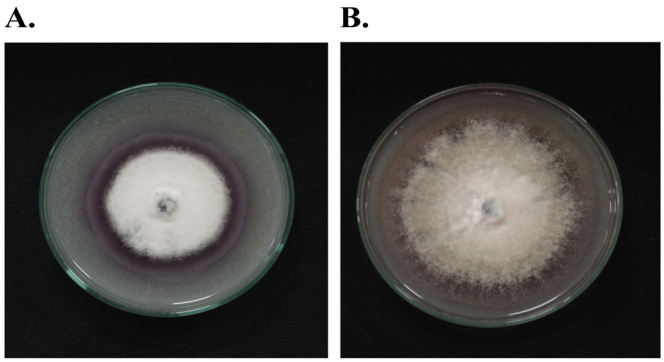


Figure 5. Production of cercosporin in V8 culture medium. Colony of isolate M6 (*C. kikuchii*) on V8 culture medium (A), and colony of isolate M11 (*Cercospora* spp.) on V8 culture medium (B).

Conclusions

The isolates were pathogenic to *Fragaria* × *ananassa* Duch. cv. Camarosa. Morphologically, on PDA medium, the colonies showed slow growth with aerial mycelium of pinkish to grayish color.

The morphological characteristics are similar to those described for the identification of cercosporid fungi, which was confirmed by molecular characterization.

Cercospora in strawberry is caused by a complex of species of the genus *Cercospora*, among them *C. kikuchii*.

The culture medium with vegetable juice provided the best condition for growth, sporulation, and cercosporin production of *Cercospora* sp. isolates.

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Compliance with Ethical Standards

Author contributions: Conceptualization: JRTK, MFBM; Data curation: JRTK, TP, LGS; Formal analysis: JRTK, MFBM; Investigation: JRTK, MFBM, LGS, VSF, TP, IGV; Methodology: JRTK, MFBM; Project administration: JRTK, MFBM; Resources: JRTK, MFBM; Supervision: JRTK, MFBM; Validation: JRTK, MFBM, VSF; Visualization: JRTK, IGV; Writing - original draft: JRTK; Writing - review & editing: MFBM, LGS.

Table 5. Mycelial growth and sporulation of *Cercospora* spp. on different culture media.

Isolated	Medium					
	CAR		MALT		V8	
	FCD (mm)	Spores (conidia mL^{-1})	FCD (mm)	Spores (conidia mL^{-1})	FCD (mm)	Spores (conidia mL^{-1})
M6	44.82	6.25×10^3	45.16	5.94×10^4	47.39	1.12×10^5
M11	51.47	1.50×10^4	51.45	6.56×10^4	56.03	5.06×10^4

CAR - carrot culture medium; MALT - malt type culture medium; V8 - vegetable juice culture medium; FCD - final colony diameter.

Conflict of interest: The authors declare that there is no conflict of interest.

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