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ORIGINAL ARTICLE

Phenotypic and genetic variability of fungal isolates associated with the *Septoria* leaf spot disease of lettuce (*Lactuca sativa*) in Brazil

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Abstract

Septoria leaf spot is a severe disease of lettuce in tropical and subtropical regions, inducing yield/quality losses and increasing production costs. Globally, *Septoria lactucae* has been reported as the major pathogen associated with this disease. However, a complex of *Septoria* species has been also reported in association with lettuce crop around the world. Extensive surveys of *Septoria* species associated with this disease have not been conducted in the Neotropics. Therefore, the objective of the present study was to undergo a phenotypic and genetic characterization of a collection of 32 Brazilian *Septoria* isolates causing lettuce leaf spot. Morphometrical characterization of conidia and pycnidia was conducted with ten isolates obtained from three lettuce-producing regions in Brazil. In addition, partial sequences of four gene regions (β-tubulin, RPB2, actin, and calmodulin) were used in phylogenetic analyses of all 32 *Septoria* isolates. All tested isolates were pathogenic to two lettuce cultivars and morphometrically similar to each other. These isolates grouped together into a single monophyletic clade composed exclusively by genuine *S. lactucae* isolates. Therefore, *S. lactucae* was found to be the sole species associated with the Septoria leaf spot of lettuce in Brazil. The number of polymorphic sites ($n=27$), mutations ($n=190$), the level of nucleotide diversity (0.00262), and the average number of nucleotide differences (2.961) were relatively low. Notwithstanding, this genetic variability allowed the identification of 17 haplotypes amongst the 32 Brazilian isolates. This information will help to guide resistance breeding programs and establish more effective management strategies of this disease.

Keywords *Lactuca sativa* · Lettuce · Septoria leaf spot · Diversity · Phylogenetic analysis

Introduction

Lettuce (*Lactuca sativa* L.) is one of the major vegetable crops around the world (Blancard et al. 2006; FAO 2017). Septoria leaf spot is amongst the most destructive diseases of lettuce in tropical and subtropical regions, being able to induce significant yield/quality losses and increasing production costs. Symptoms initiate on basal (older) leaves, and they consist of small, irregular chlorotic spots. Numerous fruiting bodies (=pycnidia) develop on the leaf spots. Under favorable environmental conditions (20–24 °C and high humidity), this disease may cause complete destruction of the foliage, particularly under heavy rainfall, frequent dew deposition or under over-head irrigation (Blancard et al. 2006; Nao 2008). After disease onset and establishment, lesions enlarge and turn brown, and premature dropping of collapsed leaves can occur. Although the disease affects primarily the leaves, the stems and the floral organs may also be damaged in lettuce fields intended for commercial seed production. Although fungicides are somewhat effective in controlling this disease, resistant cultivars would be the most economically viable control strategy due to the costs involved in the chemical management and its associated environmental risks. However, most of the leading lettuce cultivars are susceptible to *S. lactucae* (Sousa et al. 2003; Blancard et al. 2006).

Globally, *Septoria lactucae* Pass. (Mycosphaerellales) has been reported as the major pathogen associated with this disease of lettuce. This fungal species was described by Passerini in 1879, and this report was subsequently followed by synonyms such as *S. lactucae* Peck, *Aschochya lactucae*

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Rostrup, and *S. consimilis* Ellis & Martin (Punithaligam and Holliday 1972). Epidemics of *S. lactucae* have been reported in some important lettuce-producing areas in Australia, England, USA, India, China, Venezuela, Germany, Guatemala, South Africa, Ethiopia, Taiwan, Tanzania, Mexico, the Netherlands, Panama, Colombia, and Bulgaria (Farr and Rossman 2019).

Besides *S. lactucae*, a complex of *Septoria* species has also been reported in association with lettuce crop across distinct geographical regions (Bedlan 1999; Blancard et al. 2006; Lohmeir et al. 2013). Septoria leaf spot of lettuce caused by *S. birgitae* Bedlan was reported in Austria and Germany (Bedlan 1999; Lohmeir et al. 2013). *Septoria birgitae* infection is more intense under environmental conditions similar to that favorable to *S. lactucae*. Field discrimination between diseases caused by *S. birgitae* and *S. lactucae* is difficult, since both pathogens induce similar leaf symptoms in susceptible lettuce cultivars (Lohmeir et al. 2013). In addition to these two pathogens, a distinct set of *Septoria* species has been reported in association with lettuce crops, including *S. ludiviciana*, *S. fernandezii*, *S. schebelli*, *S. unicolor*, *S. sikangensis*, and *S. lactucina* (Blancard et al. 2006). In other plant pathosystems, the presence of two or more *Septoria* species infecting the same host is a common feature, as for example the leaf spot of pistachio (*Pistacia vera* L.), which is caused by two fungal species: *S. pistaciae* and *S. pistaciarum* (Crous et al. 2013). However, in the case of the lettuce crop worldwide, the role of these individual *Septoria* species as causal agents of this leaf spot disease is still unclear.

The current identification of *Zymoseptoria* and *Septoria* species is done by a combination of morphological traits and molecular phylogeny based upon the sequence analysis of four major genes, such as *β*-tubulin (*β*-TUB), calmodulin (CAL), RNA polymerase II second largest subunit (RPB2), and actin (ACT) (Quaedvlieg et al. 2011; 2013; Verkley et al. 2013). As Septoria leaf spot causes severe losses, the accurate identification of the causal agent is crucial to establish suitable management strategies for disease control. From the standpoint of the lettuce-breeding programs, the elucidation of the taxonomic status of *Septoria* species involved in this disease will guide effective methods for a proper identification of resistance sources.

So far only *S. lactucae* has been reported to be associated with Septoria leaf spot outbreaks on lettuce in Brazil (Sousa et al. 2003), but no detailed characterization has been done in order to confirm this fungal identification. In fact, extensive surveys on the diversity of *Septoria* species infecting lettuce were not yet carried out in the Neotropics. In this context, the major objective of the present study was to undergo a phenotypic and genetic characterization of a collection of 32 *Septoria* isolates associated with the lettuce leaf spot in Brazil.

Material and methods

Collection of *Septoria* isolates from lettuce

Thirty-two *Septoria* isolates were obtained from lettuce plants displaying typical symptoms of Septoria leaf spot. The diseased plants were collected during the years of 2014 and 2015 in Goiás State and in the Federal District (in central Brazil) and also in the southern State of Rio Grande do Sul (Table 1). Leaves with disease symptoms were maintained in moist chambers for 24 h. Picnidia were transferred to potato dextrose-agar supplemented with 20 mg/L tetracycline (PDA + T). Petri dishes were maintained at 23ºC under fluorescent lamps (8 h light and 16 h dark) until sporulation. For each isolate, a single germinated conidium was transferred to PDA + T under same conditions aiming to induce profuse sporulation. All fungal isolates were maintained in the Plant Pathogenic Fungi and Oomycetes collection of the National Center for Vegetable Crops Research (CNPH). The isolates are preserved by glass tubes filled with sterile distilled water (Castellani 1939) and at −80 °C (Dingra and Sinclair 1995).

Pathogenicity bioassays

Ten isolates (representative of each geographic region) were randomly selected for use in pathogenicity bioassays. For these experiments, lettuce seeds (cv. Vanda) were sown in 128 cell Styrofoam trays filled with Bioplant® (Bioplant Ltda., Nova Ponte-MG, Brazil) sterile substrate under greenhouse conditions. Fifteen days after sowing, the seedlings were transplanted into plastic pots (0.5 L), containing the sterile substrate, plus sterile soil and Osmocote 15-9-12® fertilizer (ICL Ltda., São Paulo, Brazil). Plants were inoculated 30 days after sowing. For spore suspension preparation, the isolates were grown in Petri plates containing oat meal (OM) medium, which were then placed into a BOD incubator at 23 °C and 12 h light for 15 days. The inoculum suspension was prepared for each isolate with a bristle brush and sterile distilled water, which helped remove the spore masses out of the plates. The suspension was subsequently filtered through double-layer gauze and then calibrated (with the aid of a Neubauer chamber) to a concentration of 2×10^5 conidia per mL. About 50 µL per liter of Tween 20® was added to the suspension in order to promote spore adhesion to the inoculated leaves. The isolates were inoculated by spraying the leaves of the lettuce cultivar 'Vanda' until the beginning of the suspension runoff. After inoculation, the plants were kept in a humid chamber condition (23 ± 2 °C for 48 h). Control plants were sprayed only with sterile

Table 1 List of *Septoria* species, isolate code, geographic origin in Brazil, haplotype and GenBank accession number of the β-tubulin (β-TUB), calmodulin (CAL), RNA polymerase II second largest subunit (RPB2), and actin (ACT) genes

Septoria and pseudocercospora species	Isolate code	Geographic origin	Haplotype	GenBank accession number			
				CAL	ACT	RPB2	β-TUB
S. lactucae	Sep2 (TRA 063,476)*	Distrito Federal	H05	MN698300	MN698386	MN794274	MN698334
S. lactucae	Sep3 (TRA 063,477)	Distrito Federal	H09	MN698299	MN698363	MN794275	MN698332
S. lactucae	Sep5 (TRA 063,478)	Distrito Federal	H02	MN698301	MN698387	MN794276	MN698335
S. lactucae	Sep7 (TRA 063,479)	Distrito Federal	H16	MN698302	MN698364	MN794277	MN698336
S. lactucae	Sep8 (TRA 063,480)	Distrito Federal	H17	MN698303	MN698365	MN794278	MN698337
S. lactucae	Sep10 (TRA 063,481)	Distrito Federal	H01	MN698304	MN698366	MN794279	MN698338
S. lactucae	Sep13 (TRA 063,482)	Goiás	H02	MN698305	MN698367	MN794280	MN698339
S. lactucae	Sep14 (TRA 063,483)	Distrito Federal	H03	MN698306	MN698394	MN794303	MN698340
S. lactucae	Sep17 (TRA 063,484)	Distrito Federal	H02	MN698307	MN698368	MN794281	MN698341
S. lactucae	Sep19 (TRA 063,485)	Distrito Federal	H04	MN698308	MN698369	MN794298	MN698331
S. lactucae	Sep21 (TRA 063,486)	Rio Grande do Sul	H04	MN698309	MN698370	MN794299	MN698342
S. lactucae	Sep23 (TRA 063,487)	Goiás	H06	MN698310	MN698371	MN794282	MN698343
S. lactucae	Sep24 (TRA 063,488)	Rio Grande do Sul	H07	MN698311	MN698388	MN794283	MN698361
S. lactucae	Sep25 (TRA 063,489)	Goiás	H04	MN698312	MN698372	MN794300	MN698344
S. lactucae	Sep26 (TRA 063,490)	Goiás	H02	MN698313	MN698373	MN794284	MN698345
S. lactucae	Sep28 (TRA 063,491)	Distrito Federal	H02	MN698314	MN698374	MN794301	MN698346
S. lactucae	Sep29 (TRA 063,492)	Goiás	H08	MN698315	MN698375	MN794285	MN698347
S. lactucae	Sep30 (TRA 063,493)	Goiás	H10	MN698316	MN698376	MN794286	MN698348
S. lactucae	Sep32 (TRA 063,494)	Distrito Federal	H11	MN698317	MN698377	MN794287	MN698362
S. lactucae	Sep33 (TRA 063,495)	Goiás	H04	MN698318	MN698378	MN794304	MN698349
S. lactucae	Sep34 (TRA 063,496)	Goiás	H07	MN698319	MN698389	MN794288	MN698350
S. lactucae	Sep35 (TRA 063,497)	Distrito Federal	H07	MN698320	MN698390	MN794289	MN698351
S. lactucae	Sep36 (TRA 063,498)	Goiás	H05	MN698321	MN698391	MN794290	MN698352
S. lactucae	Sep37 (TRA 063,499)	Distrito Federal	H12	MN698322	MN698379	MN794291	MN698353
S. lactucae	Sep38 (TRA 063,500)	Goiás	H02	MN698323	MN698380	MN794305	MN698354
S. lactucae	Sep39 (TRA 063,501)	Distrito Federal	H05	MN698324	MN698392	MN794292	MN698355
S. lactucae	Sep40 (TRA 063,502)	Distrito Federal	H05	MN698325	MN698381	MN794293	MN698356
S. lactucae	Sep42 (TRA 063,503)	Distrito Federal	H13	MN698326	MN698382	MN794294	MN698357
S. lactucae	Sep43 (TRA 063,504)	Distrito Federal	H14	MN698327	MN698383	MN794295	MN698333
S. lactucae	Sep44 (TRA 063,505)	Distrito Federal	H07	MN698328	MN698384	MN794296	MN698358
S. lactucae	Sep45 (TRA 063,506)	Rio Grande do Sul	H15	MN698329	MN698385	MN794302	MN698359
S. lactucae	Sep46 (TRA 063,507)	Rio Grande do Sul	H02	MN698330	MN698393	MN794297	MN698360
S. tinctoriae	CBS129154	South Korea	-	KF253046	KF252571	KF253879	KF254230
S. taraxaci	CBS 567.75	Armenia	-	KF253045	KF252570	KF253878	KF254229
S. sonchi	CBS 128,757	South Korea	-	KF253020	KF252546	KF253855	KF254204
S. sigesbeckiae	CBS 128,661	South Korea	-	KF253015	KF252541	KF253850	KF254199
S. senecionis	CBS 102,381	The Netherlands	-	KF253013	KF252539	KF253848	KF254197
S. putrida	CBS 109,087	Austria	-	KF252996	KF252521	KF253831	KF254180
S. protearum	CBS 410.61	Italy	-	KF252988	KF252514	KF253823	KF254172
S. posoniensis	CBS 128,645	South Korea	-	KF252977	KF252503	KF253811	KF254160
S. obesa	CBS 128,623	South Korea	-	KF252952	KF252477	KF253785	KF254133
S. matricariae	CBS 109,000	The Netherlands	-	KF252942	KF252468	KF253775	KF254123
S. leucanthemi	CBS 109,091	Austria	-	KF252928	KF252453	KF253760	KF254108
S. lactucae	CBS 108,943	The Netherlands	-	KF252911	KF252436	KF253743	KF254091
S. lactucae	CBS 352.58	Germany	-	KF252912	KF252437	KF253744	KF254092
S. helianthi	CBS 123.81	South Korea	-	KF252903	KF252428	KF253735	KF254083
S. helianthicola	CBS 122.81	South Korea	-	KF252904	KF252429	KF253736	KF254084

Table 1 (continued)

Septoria and pseudocercospora species	Isolate code	Geographic origin	Haplotype	GenBank accession number			
				CAL	ACT	RPB2	*β*-TUB
S. exotica	CBS 163.78	New Zealand	-	KF252890	KF252416	KF253722	KF254070
S. erigerontis	CBS 131,893;	South Korea	-	KF252888	KF252414	KF253720	KF254068
S. epambrosiae	CBS 128,629	South Korea	-	KF252880	KF252407	KF253713	KF254061
S. dysentericae	CBS 128,637	South Korea	-	KF252875	KF252404	KF253708	KF254056
S. crepidis	CBS 128,619	South Korea	-	KF252863	KF252392	KF253695	KF254043
S. cirsii	CBS 128,621	South Korea	-	KF252853	KF252382	KF253685	KF254033
S. chrysanthemella	CBS 354.73	New Zealand	-	KF252852	KF252381	KF253684	KF254032
S. chromolaenae	CBS 113,373	Cuba	-	KF252846	KF252375	KF253678	KF254026
S. chamaecisti	CBS 350.58	Germany	-	KF252843	KF252372	KF253675	KF254023
S. callistephi	CBS 128,590	South Korea	-	KF252830	KF252359	KF253662	KF254010
S. atropurpurea	CBS 348.58	Germany	-	KF252824	KF252353	KF253656	KF254004
S. astericola	CBS 128,587	South Korea	-	KF252818	KF252347	KF253650	KF253998
P. pyracanthigena	CBS 112,032	South Korea	-	KF252797	KF252324	KF253627	KF253975

*The isolate code of *Septoria lactucae* in parenthesis is the culture repository from the fungal collection of the Embrapa Hortaliças recorded in the AleloMicro System (http://alelomicro.cenargen.embrapa.br/InterMicro/index.xjs)

distilled water plus Tween 20®. Four pots (with one plant each) were used as experimental units for each isolate. Plants inoculated with distinct isolates were kept apart in order to avoid cross-contaminations. A second assay, using the same methodology, was carried out to text the pathogenicity of the ten isolates, in this time in the two lettuce cultivars 'Vanda' (crisphead) and 'Aurelia' (butterhead). Evaluation was done 10 days after inoculation recording the symptomatic and asymptomatic plants (qualitative). One symptomatic leaf of each plant was collected and taken to the Plant Pathology Laboratory and placed in moist chambers for one day. The presence of picnidia and spore cirrus on the lesions was observed under a stereoscopic microscope. Picnidia and/or spore cirrus was transferred to PDA + T plates for re-isolation of the pathogen.

Morphometrical characterization

Predominant colony color, mycelial growth, and pigmentation were evaluated for each of the 32 monosporic isolates. For morphometrical characterization of conidia and pycnidia, symptomatic leaves were collected from the plants inoculated with the ten selected *Septoria* isolates as previously described (see "Pathogenicity bioassays" section). Sections of the diseased leaves were initially observed under a Leica 205C stereomicroscope (Leica Biosystems, Nussloch GmbH, Nussloch, Germany) for presence of picnidia. Selected lesions were sectioned in a Leica CM 1850 freezing microtome producing 15–20-µm-thick fragments which were mounted in colorless lactoglycerol on glass slides and visualized using Nomarski's interference optics with a Leica DM 2500 microscope and Leica DFC 490 digital camera (Leica Biosystems, Nussloch GmbH, Nussloch, Germany). The diameter, width and length of ten picnidia and the width and length of 30 conidia were measured for each isolate (Table 2).

DNA isolation, PCR and sequencing

Thirty-two isolates were analyzed for the nucleotide sequence variation of the regions ACT, CAL, β-TUB, and RPB2. The total DNA of the fungal isolates was extracted using a modified CTAB 2X (pH = 8.0) and chloroform/isoamyl alcohol protocol (Boiteux et al. 1999). Purified DNA was stored in microcentrifuge tubes containing Tris–EDTA buffer (10-mM Tris Base and 0.5 mM EDTA solution) at −20 °C. PCR assays were performed using the set of primers

Table 2 Morphological characters of *Septoria* isolates obtained from lettuce in Brazil

Isolate code	Conidial size		Pycnidia
	Length (µm)	Width (µm)	diameter (µm)
Sep2	41.42	3.38	174.19
Sep3	44.80	3.38	140.73
Sep5	32.57	4.74	123.33
Sep7	31.76	3.58	138.30
Sep8	33.75	3.58	119.48
Sep10	38.87	4.26	137.09
Sep13	33,76	3.86	130.01
Sep14	47.72	2.99	141.6
Sep17	33.57	3.4	125.5
Sep21	38.85	3.52	160.1

listed in Table 3. Reactions were carried out in a total volume of 20 µL consisting of 2 µL of fungal genomic DNA (50 ng/µL), 2 µL 10X buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 0.6 µL $MgCl_2$ (50 mM), 1.0 µL dNTPs (2.5 M), 0.3 µL of Invitrogen® *Taq* DNA polymerase (5 units/µL), 1.5 µL of each primer, and 11.1 µL Milli-Q® water (Millipore Co., Bedford–MA, USA). The PCR conditions were established for ACT, CAL, *β*-TUB, and RPB2 essentially as described by Verkley et al. (2013) with an initial denaturation step (96 °C for 2 min) followed by 40 cycles of 96 °C for 45 s, 58 °C for 30 s, 72 °C for 1.0 min, and a final extension step at 72 °C for 2 min. Amplicons were analyzed in agarose gel (1%) electrophoresis, stained with ethidium bromide, and visualized under UV light. Sanger sequencing was done employing an ABI 3100 (Applied Biosystems, Foster City–CA) sequencer of the Genomic Analysis Laboratory at CNPH (Brasília–DF, Brazil) using the kit ABI Prism BigDye® version 3.1 (Applied Biosystems, Foster City–CA). Sequencing was performed in both forward and reverse directions in order to increase the accuracy of the final nucleotide readings.

Phylogenetic and haplotype network analyses

The reads of the four regions were assembled and edited according to their base pair quality using the Lasergene Molecular Biology Package (DNAStar, Madison–WI, USA). Ambiguities and other errors were verified in the corresponding electropherograms, which were subsequently either removed or manually adjusted. All novel sequences obtained in the present work were deposited at the GenBank database (Table 1). Sequences of all available *S. lactucae* isolates plus a subset of accessions of Asteraceae-infecting *Septoria* isolates as well as the fungal isolate selected as out-group (=*Pseudocercospora pyracanthigena*) were retrieved from the GenBank. A dataset of 227, 353, 315, and 270 bp was obtained for ACT, CAL, *β*-TUB, and RPB2 genes, respectively. The multiple sequence alignments of the obtained ACT, CAL, *β*-TUB, and RBPB2 sequences were performed with MAFFT plugin (Katoh and Standley 2013) in Geneious R8. Alignments from this study were submitted to TreeBase, and the accession information can be accessed in: http://purl.org/phylo/treebase/phylows/study/TB2:S27863?x-access-code=f169df28a317d6873594b495552e134c&format=html**.** The phylogenetic analyses were carried out for each gene individually and also by concatenating the sequences of all four regions. The substitution models were chosen for each alignment with the MEGA X software (Tamura et al. 2013). The analysis by Bayesian inference was performed on MrBayes (Huelsenbeck and Ronquist 2001) plugin (version 3.2.2) in Geneious R8 with Hasegawa–Kishino–Yano (HKY) substitution model (Hasegawa et al. 1985), plus gamma and invariable sites. Four million generations chain and 25% burn-in were applied. Only the Bayesian inference of concatenated sequences is presented here due to the high similarity levels of the tree topologies obtained with information from the ACT, CAL, *β*-TUB, and RBPB2 regions. The number of haplotypes from the four loci for the *S. lactucae* populations was calculated using the software DnaSP v5 (Librado and Rozas 2009). A haplotype network for concatenated sequences was reconstructed with PopART (Leigh and Bryant 2015) using the TCS algorithm (Clement et al. 2002) with gaps and missing data excluded.

Results

Pathogenicity bioassays

All ten inoculated isolates were pathogenic to 'Vanda' lettuce plants under greenhouse conditions in the first assay. In the second assay, all isolates were also pathogenic to both lettuce cultivars ('Vanda' and 'Aurelia'). No detectable variation in aggressivity was observed among isolates in both cultivars with all of them inducing lesions with pycnidia within 13–15 days after inoculation. Symptoms were identical to those found in field-infected plants, displaying conspicuous leaf spots (Fig. 1). Control plants were free of symptoms throughout the assays. All isolates were reisolated from the inoculated plants fulfilling the Koch's postulates.

Table 3 Primer combinations used to amplify and sequence *β*-tubulin (*β*-TUB), calmodulin (CAL), RNA polymerase II second largest subunit (RPB2), and actin (ACT) genes of *Septoria* isolates obtained from lettuce leaf spots in Brazil

Genomic region	Primer	Primer sequence 5' to 3'	Reference
β-TUB	T1	AACATGCGTGAGATTGTAAGT	Verkley et al. (2013)
	β-Sandy-R	GCRCGNGGVACRTACTTGTT	Stukenbrock et al. (2012)
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)
	fRPB2-414R	ACMANNCCCCARTGNGWRTTRTG	Quaedvlieg et al. (2011)
ACT	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)
	ACT2Rd	ARRTCRCGDCCRGCCATGTC	Verkley et al. (2013)
CAL	CAL-235F	TTCAAGGAGGCCTTCTCCCTCTT	Quaedvlieg et al. (2012)
	CAL2Rd	TGRTCNGCCTCDCGGATCATCTC	Verkley et al. (2013)

The PCR conditions were established for ACT, CAL, *β*-TUB, and RPB2 essentially as described by Verkley et al. (2013). The annealing temperature for all primer pairs was 58 °C

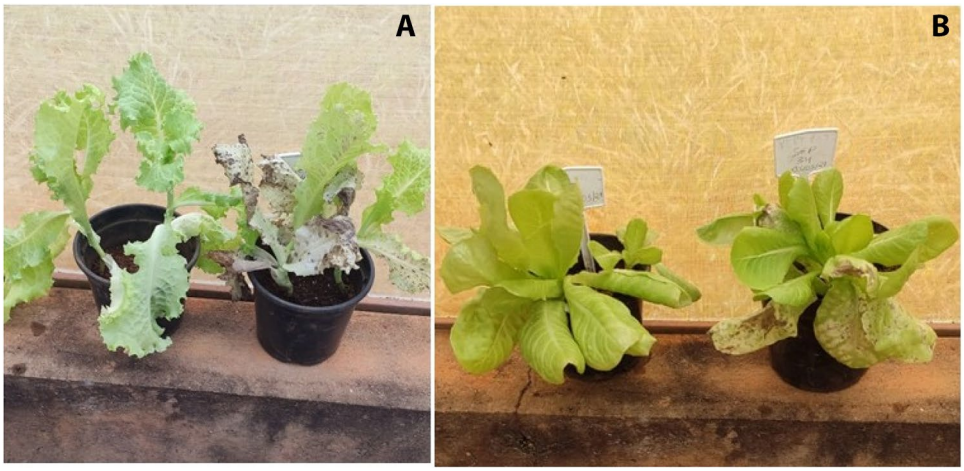

Fig. 1 Symptoms of Septoria leaf spot on two lettuce cultivars: Vanda (**A**) and Aurélia (**B**) induced by the isolates Sep10 and Sep21, respectively, inoculated under greenhouse conditions (left = controls)

Morphometrical characterization

The Brazilian *Septoria* isolates from lettuce analyzed in the present study displayed similar morphological attributes of the colonies and also a quite similar range of morphometrical variation of pycnidia and conidia. In the OA medium, the isolates produced colonies varying from 8 to 10 mm in diameter in about 2 weeks, with an even to undulating, colorless margin; colonies spreading to restricted, immersed mycelium pale luteous, with low amount of aerial mycelium. Pycnidia developed immersed on the agar surface, mostly in the center of the colonies or in radiating rows. Milky white conidial masses were released from some pycnidia. Brazilian isolates displayed filiform conidia, 1–3 septate, measuring 31.76–47.42 × 2.99–4.74 µm and with average pycnidium diameter range of 137.09–174.19 µm (Table 2). In the microtome, sectioned lesion tissue picnidia with an approximately spherical format were observed. Conidia were similar to those observed in culture (Fig. 2). All these morphological and morphometrical characteristics were in agreement with the description of *S. lactucae* (Verkley et al. 2013).

Phylogenetic analyses

The phylogenetic analyses of the concatenated sequences of 1138 bp indicated a consistent single-cluster pattern of the *S. lactucae* isolates from distinct lettuce-producing areas of Brazil (Fig. 3). The isolates from Brazil grouped with two *S. lactucae* isolates from the Netherlands and Germany as well as with an isolate of *S. sonchi* with posterior probability of 1.0. Three subclades were formed in the phylogenetic tree (Fig. 3). Two of them were composed by Brazilian isolates collected across distinct geographical areas. The isolates from the Netherlands and Germany grouped with *S. sonchi* in another subclade (Fig. 3).

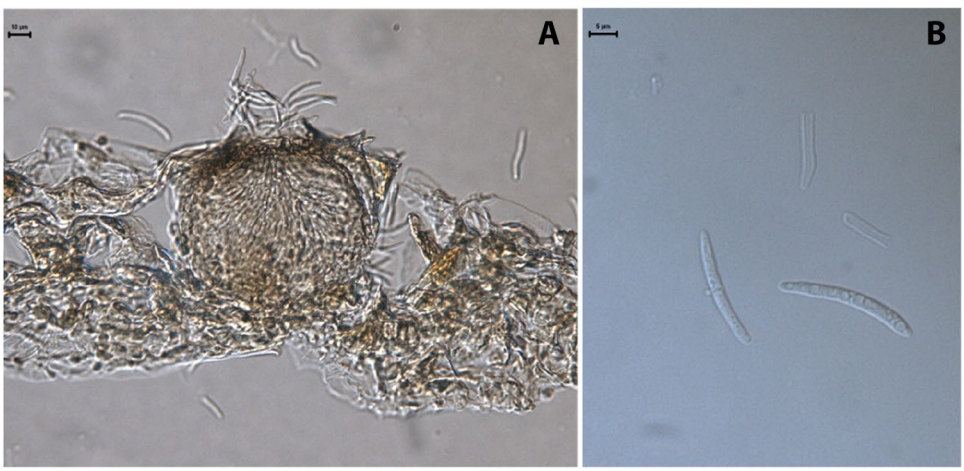

Fig. 2 Reproductive structures of *Septoria lactucae* observed under light microscope. Pycnidium (**A**) and Conidia (**B**)

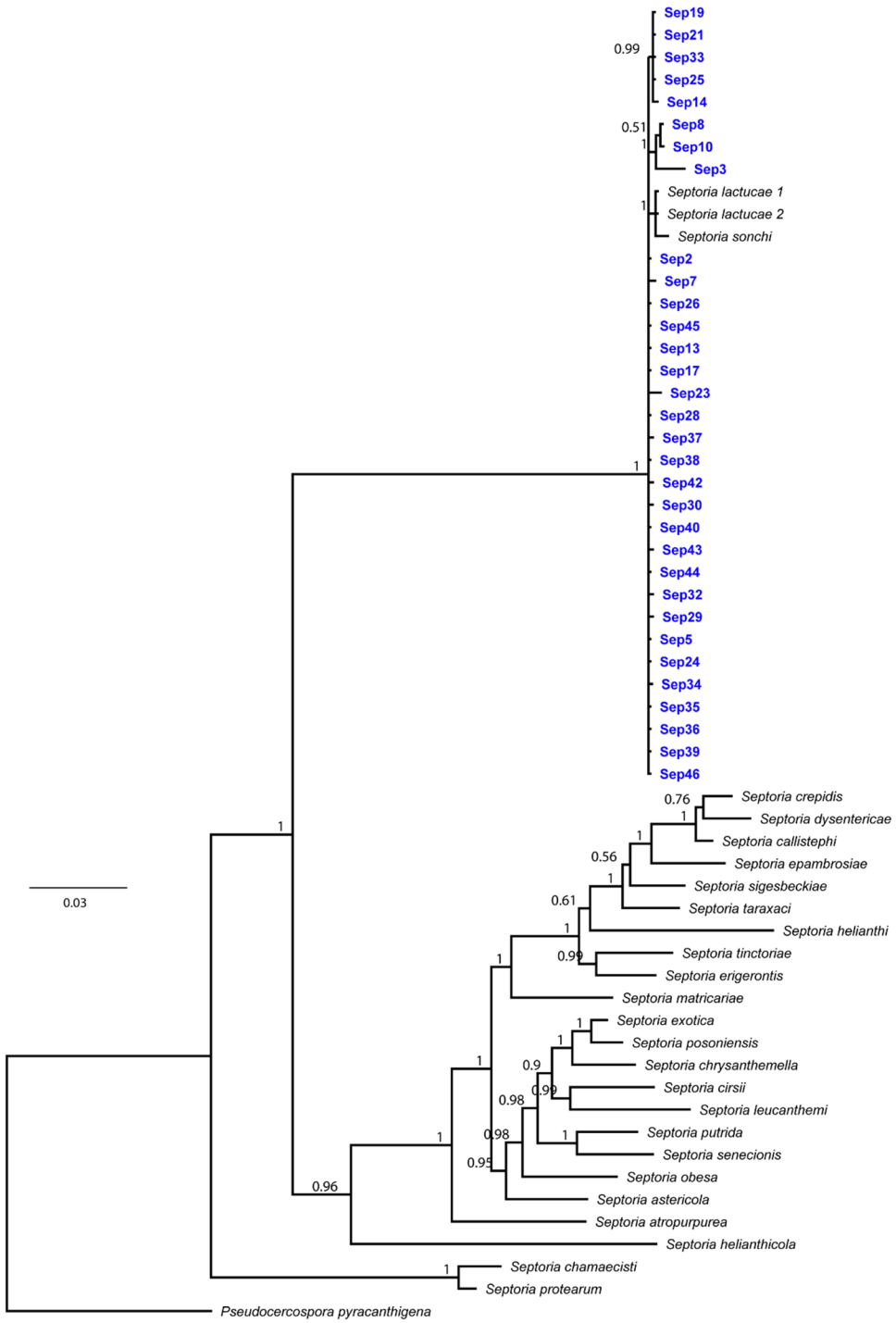

Fig. 3 Phylogenetic tree of concatenated partial sequences of the β-tubulin (β-TUB), calmodulin (CAL), RNA polymerase II second largest subunit (RPB2), and actin (ACT) genes from *Septoria* species along 32 Brazilian isolates by Bayesian inference with HKY + I + G model with four million generations and 25% burn in with *Pseudocercospora pyracanthigena* as out-group

Haplotype network

The haplotype network was established according to the Bayesian inference topological tree. The number of polymorphic sites, mutations, and haplotypes was 27, 19, and 17, respectively. For the nucleotide diversity and average number of nucleotide differences, the estimated values were 0.00262 and 2.961, respectively. There is one discernible central haplotype from which most other haplotypes diverged (Fig. 4). Several haplotypes were unique, and they were represented by only one sequence (Fig. 4). Sequences from Federal District, Goiás State, and Rio Grande do Sul State were represented in 12, seven, and four haplotypes, respectively (Table 1). The Federal District displayed the highest number of unique haplotypes in comparison with other geographical locations. The most diverse and frequent haplotype was H02,

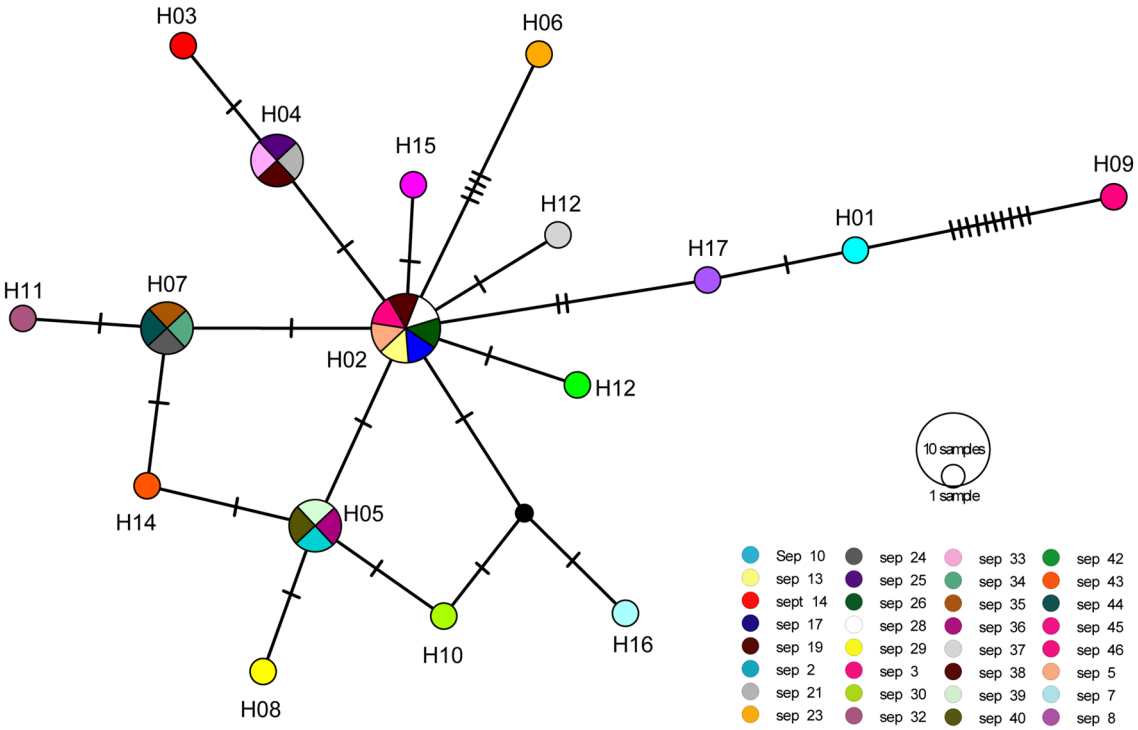

Fig. 4 TCS haplotype networks of the concatenated partial sequences of the *β*-tubulin (β-TUB), calmodulin (CAL), RNA polymerase II second largest subunit (RPB2), and actin (ACT) genes from *Septoria* isolaters collected from lettuce plants in Brazil, generated using PopArt. Each color represents haplotypes. The sizes of the circles are proportional to the frequency of each haplotype. Black circles represent hypothetical haplotypes. Hatch marks along the network branches indicate the number of mutations

which was represented by sequences of isolates from the Federal District, Goiás State, and Rio Grande do Sul State (Fig. 4). Most isolates from different geographic origins were grouped in the haplotypes 2, 4, 5, and 7. Haplotype 2 included seven isolates, while four isolates were classified as haplotypes 4, 5, and 7. Pathogen populations collected in the Federal District displayed higher number of haplotypes ($n=13$), followed by Goiás State ($n=7$) and Rio Grande do Sul State ($n=4$). This higher number of pathogen haplotypes found in the Federal District can be explained by a sampling effect, since the number of isolates obtained in this Brazilian federation unit is larger ($n=18$) (Table 1).

Discussion

A complex of *Septoria* species has been reported in association with lettuce crop across the world (Bedlan 1999; Blancard et al. 2006; Lohmeir et al. 2013). However, no extensive studies were carried out thus far to estimate the diversity of *Septoria* species infecting lettuce in the Neotropics. In the present work, a phenotypic and genetic characterization of fungal isolates associated with the lettuce leaf spot in Brazil was conducted aiming to clarify the panorama of the potential causal agents of this disease under Brazilian conditions. Our study was based on a combination of morphological characteristics as well as phylogenetic analysis using the nucleotide information of four regions.

Morphological differences for conidia size, pycnidia diameter, mycelial growth, and aspects of the colonies of the isolates used in our study were all in the range described for the species *S. lactucae* (Verkley et al. 2013). Additional confirmation was obtained via phylogenetic analysis of the concatenated sequence data of the *β*-TUB, ACT, CAL, and RPB2 amplified from 32 lettuce-infecting *Septoria* isolates from Brazil, which indicated that all belong to the species *S. lactucae*.

The level of DNA polymorphism in sequences was relatively low. Nevertheless, this nucleotide dataset allowed for the establishment of more precise phylogenetic relationships among the *S. lactucae* isolates. Low genetic diversity is consistent with predominantly asexual reproduction as also inferred for *Alternaria* species in Brazil (Lourenço Jr. et al. 2009; Peixoto et al. 2021). In the study of Lourenço Jr. et al. (2009) were detected low number of mutations ($n=16$), nucleotide diversity (0.007), and nucleotide differences (3.20) for *Alt a1* gene. As the sexual morph is unknown in *S. lactucae*, further investigation about the mechanisms of reproduction in this species should be carried out.

The genetic variability of the four regions analyzed in our study allowed for the identification of 17 haplotypes amongst the 32 Brazilian *S. lactucae* isolates. The majority of the haplotypes was composed by a single isolate. No clear association of the isolate haplotype with geographic area was detected as indicated by the arrangements of clades and subclades, which were composed by isolates sampled across all locations. As *S. lactucae* is a seed-borne plant pathogen, the fungus dispersal takes place mainly by passive movement of infected seeds at short and long distances locally or even across large geographical areas (Bertus 1972; Blancard et al. 2006). To assess properly the importance of migration and other evolutionary processes that shape the *S. lactucae* population in Brazil, population genetic studies based on a set of molecular markers for a large number of genes should be conducted with a higher number of isolates.

Although *S. sonchi* clustered in the same clade of *S. lactucae*, there is no report in the literature of this plant pathogen associated with Septoria leaf spot on lettuce. In addition, *S. sonchi* has not been detected in Brazil. *Septoria sonchi* is morphologically distinct from *S. lactucae*, producing pycnidia on leaf tissue with 60–100 µm in diameter and conidia measuring 15–30 × 1.5–2.0 µm (Zafari and Razagui 2013). In a separate study, *S. sonchi* isolated from the host *Sonchus asper* in South Korea clustered in the same clade of two *S. lactucae* isolates with 100% of bootstrap in the Bayesian analysis of the combined loci of translation elongation factor-1α, LSU, ITS, *β*-TUB, ACT, CAL, and RPB2 (Verkley et al. 2013). Therefore, *S. lactucae* and *S. sonchi* are closely related, and the gene regions used in both studies are conserved between the two species. Other genes should be evaluated in further phylogenetic studies in order to discriminate the *S. lactucae* from the *S. sonchi* isolates. Another interesting approach is to assess the pathogenicity of *S. sonchi* on lettuce.

Septoria birgitae was identified in Austria and Germany associated with Septoria leaf spot of lettuce (Bedlan 1999; Lohmeir et al. 2013). This species differs in the diameter of the pycnidia (85.96–195.75 µm), length (16.25–36.73 µm), width (1.28–2.30 µm), and number of septa (1–3) of the conidia (Bedlan 1999). However, there is no DNA sequences of this specimen deposited in public databases such as GenBank. This fact made it impossible to include this species in the phylogenetic analyses performed in our study. The information about other *Septoria* species found on lettuce (Blancard et al. 2006) is unclear, and there is no recent report about the occurrence of these fungi in this crop.

The development of cultivars with high levels of resistance to *Septoria* species is a major lettuce-breeding objective. For a stable resistance, it is desirable to obtain lettuce cultivars with large spectrum resistance to all different *S. lactucae* haplotypes as well as other potential lettuce-infecting *Septoria* species. In this context, the identification of a single *Septoria* species associated with the disease may facilitate the establishment of effective germplasm screening for potential sources of resistance under Brazilian conditions. Besides, additional pathogenicity and aggressiveness bioassays using isolates from different *S. lactucae* haplotypes found in our study should be carried out on lettuce germplasm accessions aiming to select more suitable fungal isolates for resistance screenings. Partial resistance was detected in the cultivars 'Simpson,' 'Mimosa,' 'Babá de Verão,' and 'Vitória de Santo Antão' in Brazil under greenhouse and field experiments (Sousa et al. 2003). Additional studies on the population biology, fungicide sensitivity, and epidemiology are also needed to develop suitable management strategies aiming to reduce the impact of this disease on the lettuce yield and quality.

Globally, *S. lactucae* and also a complex of *Septoria* species have been reported as the major pathogen associated with this disease in lettuce. However, this complex seems to be not relevant under Brazilian conditions. All isolates (collected across geographically distinct lettuce-producing areas) were morphometrically similar, and they grouped together into a single clade composed exclusively by genuine *S. lactucae* isolates. The number of polymorphic sites ($n = 18$), mutations ($n = 20$), the level of nucleotide diversity (0.00160), and the average number of nucleotide differences (1.78) were relatively low. Notwithstanding, this genetic variability allowed the identification of 17 haplotypes amongst the 32 Brazilian isolates. In conclusion, *S. lactucae* was found to be the sole species associated with the Septoria leaf spot of lettuce in Brazil. To our knowledge, this is the first study of morphological and molecular characterization of the fungal species associated with Septoria leaf spot on lettuce in the Neotropics. This information will help to guide resistance breeding programs and for establishing more effective disease management strategies.

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Declarations

Conflict of interest The authors do not have any conflict of interest.

Data availability Alignments from this study were submitted to TreeBase and the sequences to GenBank.

Informed consent All authors have reviewed the manuscript and approved its submission to Journal of Plant Disease and Protection.

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