DNA barcoding, aggressiveness of Bipolaris sorokiniana isolates, and pathogenicity of emerging B. gossypina in barley in subtropical southern Brazil

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Abstract

Southern Brazil has experienced severe outbreaks of leaf blotch disease in barley, which have led to reduced grain quality and yield. A field survey was conducted in the major barley-producing areas of Paraná state using DNA barcoding techniques to identify *Bipolaris sorokiniana* isolates, the causative agent of spot blotch, aiming to determine the extent of pathogenic variability among the isolates. DNA barcoding and phylogenetic analyses were based on internal transcribed spacers 1 and 2 with the 5.8S region (ITS) of rDNA, glyceraldehyde-3-phosphate dehydrogenase (gapdh), and translation elongation factor 1-α (tef1) genes. Out of the 124 isolates examined, which were collected from 20 commercial barley fields each year in 2020 and 2021, 116 isolates were identified as *B. sorokiniana* and eight isolates as *B. gossypina*. Koch's postulates confirmed the pathogenicity of *B. gossypina* in barley, representing a novel occurrence worldwide. Previously, this pathogen was found only in cotton (*Gossypium* sp.) in Kenya. The fungus causes the development of elongated brown lesions surrounded by irregular yellow halos, starting at minute points. Subtle differences between these symptoms and spot blotch caused by *B. sorokiniana* are discussed. This study also assessed the aggressiveness of 16 *B. sorokiniana* isolates on potted barley grown under controlled conditions, using a visual infection rate (IR) scale ranging from 1 to 9. Significant differences in aggressiveness were recorded among the isolates, with the IR ranging from 5.1 to 7.4 in the cultivar ANA03 and 5.7 to 8.1 in the cultivar Imperatriz. The interaction between the isolates and cultivars was not significant. These findings could support breeding programs aiming to develop cultivars with genetic resistance to spot blotch disease in Brazil.

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most widely produced cereal crop after maize, wheat, and rice (FAOSTAT 2023). Although Brazil is not among the major producing countries in the world, the annual production of approximately 480 thousand tons in a cultivated area of 120 thousand hectares has a relevant economic impact on the country. Barley is predominantly grown in the southernmost subtropical states of Brazil as a winter-spring crop, primarily to produce malt used by the brewing industry and to a lesser extent in animal feeds. Paraná State has the largest cultivation area of barley in Brazil. The average yield over the past five years (2018−2022) was 3623 kg per hectare (CONAB 2023). However, yields can reach up to 6000 kg per hectare with advanced technologies, including proper disease management. Among the diseases that affect barley production is spot blotch, caused by the fungus *Bipolaris sorokiniana*.

Spot blotch is a major foliar disease of barley and wheat (*Triticum aestivum* L.) in the warm, humid regions of Asia, Europe, and the Americas (Kumar et al. 2002; Ghazvini 2018), including subtropical southern Brazil. Diseased leaf blades and sheaths develop light-to-dark brown blotches that are oval to elongated in shape and can merge to cover large foliar areas. Seedling blight, root rot, and black points in mature grains are caused by the same pathogen (Kumar et al. 2002). Spot blotch can cause significant yield losses, as demonstrated by studies conducted in North America and South Asia (Mathre 1997; Ghazvini 2018). In Brazil, damage can lead to yield losses as high as 49%, as reported by Agostinetto et al. (2015) under experimental conditions.

Outbreaks of spot blotch are more likely to occur during prolonged periods of leaf wetness associated with temperatures above 20°C (Mathre 1997), which are common conditions during the barley growth cycle in winter and spring in southern Brazil. Management of the disease is complex and expensive because cultural control has limited effectiveness and the genetic resistance of commercial cultivars is not sufficient to prevent the use of chemical fungicides. Four to five sprays may be needed for satisfactory control of the disease (Agostinetto et al. 2015). However, despite its limited effectiveness, genetic resistance remains the most desirable method for controlling spot blotch, considering economic and environmental sustainability.

The damage caused by foliar spots on barley crops in southern Brazil is usually more noticeable during the grain filling phase. At this stage, the spot blotch lesions merge and occupy large areas of the leaf blade, causing significant variation in disease symptoms. Additionally, these spots may overlap with symptoms of other foliar diseases, such as the net blotch caused by *Pyrenophora teres* Drechsler, forming a complex of foliar spots that are challenging to differentiate. Net blotch can be of two types: the net type, caused by *P. teres* f. *teres*, or the spot type, caused by *P. teres* f. *maculata* (Neopane et al. 2015). The extent to which the spot-type form is part of the barley leaf spot complex and is confused with spot blotch is unknown.

DNA barcoding methods have significantly improved the identification of plant pathogens (Crous et al. 2015). To identify species of *Bipolaris*, internal transcribed spacers 1 and 2 with the 5.8S region (ITS) of rDNA, as well as portions of the glyceraldehyde-3-phosphate dehydrogenase (gapdh) and translation elongation factor 1-α (tef1) genes, have been accepted as loci for DNA barcoding (Marin-Felix et al. 2017; Bhunjun et al. 2020). This has proven to be a rapid and accurate identification procedure for a large number of pathogenic isolates for subsequent pathogenic characterization or other genetic studies (Ahmadpour et al. 2018; Alkan et al. 2022; Sharma et al. 2022). In addition, new species within this genus have been described through phylogenetic characterization based on analysis of these loci (Lourenço et al. 2017; Ferdinandez et al. 2022).

Studying the pathogenic variability of *B. sorokiniana* is important for genetic breeding aimed at improving resistance to spot blotch. Resistance to spot blotch in barley is predominantly inherited through quantitative resistance (Ghazavini 2018), although there is evidence of isolate-specific interactions, indicating that qualitative resistance also plays a role (Ghazvini and Tekauz 2008; Gamba et al. 2020). Pathogenic variability in *B. sorokiniana* affecting barley has been observed in Canada, the USA, Argentina, and other countries (Fetch and Steffenson 1994; 1999; Ghazvini and Tekauz 2008; Gamba et al. 2020). However, this information is not yet available in Brazil.

This study aimed to investigate the pathogenic diversity of spot blotch causal agents in barley in Paraná State, Brazil, using DNA barcoding and phylogenetic analysis based on ITS, gapdh, and tef1 for species identification. Additionally, the extent of variation in aggressiveness among the isolates of *B. sorokiniana*, the predominant species, was examined in a controlled environment. This study also tested the pathogenicity of the emerging *B. gossypina* species in barley.

Material and Methods

Fungal isolates

Barley leaves exhibiting spot blotch symptoms were collected from 20 commercial fields that were severely damaged by spot blotch during the grain-filling stage in Paraná State, Brazil, in 2021 and 2022 (Fig. 1). In all the fields sampled, barley was grown in crop rotation with wheat or oats in winter and corn or soybeans in summer. In each field, diseased leaves were collected at five sampling points, approximately equidistant, along a 50 m line transect. The greatest distance between sampled fields was approximately 315 km. Leaf fragments of approximately 9 mm² from the transition zone between healthy and diseased tissues were collected to isolate fungi. These fragments were dipped in 50% alcohol and then transferred to 0.5% sodium hypochlorite solution for one minute. The tissue was then washed with sterile distilled water and dried using sterile filter paper. The dried leaf pieces were then placed on modified potato dextrose agar (MPDA) in 9 cm diameter Petri dishes. The MPDA used contained 62.5 g/L potato and 5 g/L dextrose, instead of the typical 200 g/L potato and 20 g/L dextrose, along with 20 g/L agar. The plates were maintained at 20 ± 2°C under a 12-h light and 12-h dark cycle until typical *Bipolaris* colonies produced conidia within

7–14 days. Single-spore isolates were grown on PDA slants and preserved on PDA slants with mineral oil at 6°C at the Plant Pathology Laboratory of the Universidade Estadual de Maringá.

Molecular identification and phylogenetic analyses

Genomic DNA was extracted from individual spore isolates grown on PDA medium for 5–7 days using a PureLink™ Genomic Plant DNA Purification Kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Initially, 120 isolates were identified and characterized based on ITS rDNA region analysis. Eighteen isolates representing different species of *Bipolaris* were further identified based on the gapdh and tef1 genes. PCR amplification was performed using the ITS4/ITS5 (White et al. 1990), gpd1/gpd2 (Berbee et al. 1999), and EF1-983F/EF1-2218R (Rehner and Buckley 2005) primers (Online Resource 1). The PCR product was purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher, USA) and sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, USA), and the sequences were analysed on an ABI3500 DNA Sequencer (Applied Biosystems, USA). For each isolate and locus, contigs were generated from the forward and reverse sequences using BioEdit version 7.2.5 (Hall 2011). The obtained sequences were compared with those in GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTn algorithm (Altschul et al. 1990). The sequences of the reference strains from the most similar species identified through BLAST searches, as validated by Marin-Felix et al. (2017), were chosen for alignment with the sequences obtained in this study. Sequences were aligned using the MUSCLE algorithm implemented in MEGA X (Kumar et al. 2018).

Phylogenetic trees were reconstructed using Bayesian and maximum likelihood methods. Bayesian inference was performed using the "MrBayes on XSEDE" tool (Ronquist et al. 2012) at the CIPRES web portal (Miller et al. 2010), applying the GTR + I + G substitution model for ITS and HKY + I for gapdh and tef1. Markov chain Monte Carlo was applied to determine posterior probabilities. Six simultaneous Markov chains were run for 10,000,000 generations and trees, and the burn-in was set at 25%. Bayesian posterior probabilities were calculated from the remaining trees. Maximum likelihood analysis was conducted using MEGA X with the K2 + G model of molecular evolution, and clade stability was assessed using bootstrap analysis with 1000 replicates. All trees were visualized in FigTree v1.4.0 (Rambaut 2009). The sequences obtained in this study were deposited in GenBank under the accession numbers listed in Table 1.

Table 1

Isolates from *Bipolaris* spp. analyzed in the study geographical coordinates of the place where they were collected in the state of Paraná, Brazil, and information on the reference strains of the species used for comparisons

Species	Isolate	Origin		ITS	GenBank accession numbers		Reference
		Latitude	Longitude	ITS	*gapdh*	*tef1*	
B. bicolor	CBS 690.96	-	-	KJ909762	KM042893	KM093776	Manamgoda et al. (2014)
B. gossypina	BRIP 14840^T	-	-	KJ415528	KJ415418	KJ415467	Tan et al. (2014)
B. gossypina	UEM4658	-51.94647	-25.92900	OQ743523	OQ834886	OQ834904	Present study
B. gossypina	UEM4662	-52.16763	-25.04637	OQ740152	OQ834887	OQ834905	Present study
B. gossypina	UEM4680	-52.01850	-25.82228	OQ743516	OQ834888	OQ834906	Present study
B. gossypina	UEM4698	-51.12008	-25.38405	OQ743518	OQ834889	OQ834907	Present study
B. secalis	BRIP 14453	-	-	KJ415537	KJ415409	KJ415455	Tan et al. (2014)
B. shoemakeri	BRIP 15929	-	-	KX452453	KX452419	KX452470	Tan et al. (2016)
B. sorokiniana	CBS 110.14	-	-	KJ922381	KM034822	KM093763	Manamgoda et al. (2014)
B. sorokiniana	UEM4610	-52.07378	-25.59318	OQ740137	OQ834872	OQ834890	Present study
B. sorokiniana	UEM4618	-52.37313	-24.54020	OQ740138	OQ834873	OQ834891	Present study
B. sorokiniana	UEM4625	-51.57185	-25.55665	OQ743509	OQ834874	OQ834892	Present study
B. sorokiniana	UEM4631	-51.65917	-25.53662	OQ743515	OQ834875	OQ834893	Present study
B. sorokiniana	UEM4639	-52.35152	-25.35688	OQ740149	OQ834876	OQ834894	Present study
B. sorokiniana	UEM4644	-51.45802	-24.48695	OQ743512	OQ834877	OQ834895	Present study
B. sorokiniana	UEM4650	-51.63417	-25.20805	OQ743514	OQ834878	OQ834896	Present study
B. sorokiniana	UEM4665	-51.68978	-24.82582	OQ743511	OQ834879	OQ834897	Present study
B. sorokiniana	UEM4674	-52.18040	-26.14260	OQ743513	OQ834880	OQ834898	Present study

Species	Isolate	Origin			GenBank accession numbers			Reference
		Latitude	Longitude	ITS	*gapdh*	*tef1*		
B. sorokiniana	UEM4687	-51.82282	-25.78728	OQ743521	OQ834881	OQ834899	Present study
B. sorokiniana	UEM4693	-52.04023	-24.93805	OQ743508	OQ834882	OQ834900	Present study
B. sorokiniana	UEM4733	-51.49113	-24.97303	OQ743522	OQ834883	OQ834901	Present study
B. sorokiniana	UEM4749	-50.14250	-25.00100	OQ743519	OQ834884	OQ834902	Present study
B. sorokiniana	UEM4751[2]	-49.80997	-24.15674	OQ743517	OQ834885	OQ834903	Present study
B. variabilis	CBS 127716	-	-	KY905676	KY905688	KY905696	Marin-Felix et al. (2017)
B. zeae	BRIP 11512	-	-	KJ415538	KJ415408	KJ415454	Tan et al. (2014)
B. woodii	BRIP 12239	-	-	KX452458	KX452424	KX4524725	Tan et al. (2016)
B. microstegii	CBS 132550	-	-	JX089579	JX089575	KM093756	Crous et al. (2012) Manamgoda et al. (2014)
B. zeicola	FIP 532	-	-	KM230398	KM034815	KM093752	Manamgoda et al. (2014)

[T] type strain.

Morphological characterization

A mycelial plug was transferred from a 7-day-old culture grown in PDA medium to a 6 cm Petri dish containing V8 agar medium (200 mL of V8 juice, 3 g of CaCO3, 17 g of agar, and 800 mL of distilled water). The plate was kept under the same temperature and lighting conditions as those described earlier. Cultural and morphological characteristics were determined using 14-day-old cultures. To measure the size of the conidia and conidiophores, the length and width of 50 randomly selected samples were measured using a Moticam 1080 camera attached to a Motic BA310E microscope with a ×40 objective. The morphological information obtained was compared to the descriptions of *Bipolaris* spp. (Sivanesan 1985; 1987).

Inoculum preparation

The inoculum was prepared using the same procedure as described for morphological characterization. Conidia were harvested from 14-day-old cultures by adding 20 mL distilled water and scraping the agar surface with a sterile rubber spatula. The concentrated spore suspension was filtered through three layers of cheesecloth to remove the mycelial fragments. Additional water was added to adjust the concentration to 5000 conidia/mL, as measured using a haemocytometer.

Preparation of plants

The barley plants were grown in pots containing a 1:1 mixture of soil and vermiculite. Each pot contained 3–4 plants with 2–3 tillers per plant. Before seeding, 0.20 g of NPK fertilizer (10:10:10) was added to each pot, followed by 0.10 g of urea 25 days after planting. Irrigation was performed to ensure an adequate water supply for normal plant growth. Potted plants were kept in a growth chamber at 22 ± 2°C with a 12-hour light and 12-hour dark cycle, using 45 W 3200 K yellow LED lamps alternating with 45 W 6500 K white LED lamps.

Pathogenicity test of B. gossypina **and aggressiveness assay of** B. sorokiniana **isolates**

The pathogenicity test for *B. gossypina* and comparison of aggressiveness of *B. sorokiniana* isolates were conducted as independent trials in a plant growth cabinet. In both trials, barley plants were inoculated at the mid-tillering stage (GS24) (Zadoks et al. 1974) by spraying approximately 20 mL per pot of a conidial suspension at a previously described concentration. The spore suspension was supplemented with 0.05% Tween 20 to ensure dispersal of the inoculum onto the leaf surfaces. The control plants were sprayed with sterile water containing 0.05% Tween 20. Inoculated plants were kept in a dew chamber at 100% relative humidity for 18 h in darkness at 20 ± 2°C, followed by an 18-h photoperiod at the same temperature. The pathogenicity test for two isolates of *B. gossypina* (UEM4658 and UEM4698) was conducted on the Imperatriz cultivar using a completely randomized design with seven replicates. Additionally, the isolate *B. sorokiniana* UEM4610 was inoculated as a positive control with a conidial suspension at the same concentration as described above. Symptom evaluations were conducted seven and 14 days after inoculation. *Bipolaris gossypina* and *B. sorokiniana* were re-isolated from the inoculated barley plants to complete Koch's postulates. The assay was completely randomized with 10 replicates. Each replicate consisted of a pot with 3–4 plants. The trial was conducted twice.

The *B. sorokiniana* aggressiveness trial was conducted using a completely randomized experimental design with a 17 × 2 factorial arrangement, 16 isolates, and one control inoculated on the ANA03 and Imperatriz cultivars, with three replications per treatment. Each pot was considered an experimental unit. The inoculation procedure and growth chamber conditions were the same as those previously described. The disease was assessed on the upper three fully expanded leaves ten days after infection using the 1–9 infection rating scale developed by Fetch and Steffenson (1999). The experiments were conducted twice.

Data analysis

Descriptive analysis of the data was performed using the boxplot function in R software (R Core Team, 2022). Shapiro–Wilk and Bartlett tests were used to assess homogeneity and homoscedasticity of variances, and there was no need for data transformation. Analysis of variance was performed using the easyanova package for R (Arnhold 2013), and the treatment means were compared using Fisher's least significant difference test with a 5% probability.

Results

Molecular characterization and species identification

The phylogenetic tree based on the ITS dataset (405 bp), which included 125 isolates of *Bipolaris* spp. associated with spot blotch, showed a major clade grouping 117 isolates with a *B. sorokiniana* CBS 110.14 reference strain, with Bayesian posterior probability (B-PP) and maximum likelihood bootstrap (ML-BS) values of 1 and 76%, respectively (Online Resource 2). Additionally, a smaller clade was observed within this group, grouping three isolates with high B-PP and ML-BS values of 0.95 and 62%, respectively. Another clade that grouped eight isolates with a *B. gossypina* BRIP 14840 type strain was identified, with B-PP and ML-BS values of 1.0 and 97%, respectively. Five isolates of *B.*

gossypina were collected in 2021 and three in 2022. These isolates were found in the field, together with isolates of *B. sorokiniana*.

After selecting a subset of 14 isolates representing *B. sorokiniana* and four isolates representing *B. gossypina*, further analyses were conducted based on the *gapdh* and *tef1*. Trees were individually inferred along with the ITS dataset for each of these datasets, and a combined analysis was also performed. The ITS tree showed a major clade grouping 14 isolates with a *B. sorokiniana* reference strain, with high B-PP and ML-BS values of 1 and 66%, respectively (Fig. 2A). A smaller clade within this group was also observed, grouping three isolates, with high B-PP and ML-BS values of 0.95 and 62%, respectively. Four isolates were also found to group with a *B. gossypina* reference strain, with high B-PP and ML-BS values of 1 and 97%, respectively. The gapdh and tef1 trees showed clades grouping isolates with reference strains of *B. sorokiniana* and *B. gossypina*, with B-PP and ML-BS values ranging from 0.76–1 and 88–99%, respectively (Fig. 2B, 2C). When the datasets were combined, both clades grouping isolates with reference strains of *B. sorokiniana* and *B. gossypina* were well-supported, with high B-PP and ML-BS values of 1 and 99%, respectively (Fig. 2D). In addition, no significant variability was observed within the *B. sorokiniana* clade based on the *gapdh* and *tef1* trees.

The analysis of morphological features of *Bipolaris gossypina* revealed that colonies growing on PDA showed a velvety layer of light grey mycelium in the centre, turning light and creamy towards the edges, and measuring 6.2 – 7.3 cm in diameter after 7 days. The colony's reverse side was dark in the centre and creamy towards the periphery, with irregular margins (Fig. 3A, 3B). Conidial production was scarce. The conidiophores were mostly single, primarily flexuous but sometimes straight, smooth, pale or brown, septate, and geniculate above, measuring 124–193 × 5–10 μm. The conidia were olivaceous brown, smooth, obclavate to subcylindrical, narrowing towards the tip, measuring 52 – 80 × 13 – 15 μm, and with 7 – 10 distosepta (Fig. 3C, 3D). These characteristics are in accordance with Sivanesan's (1985) description of the species. *Bipolaris sorokiniana* colonies grown on PDA formed a velvety layer of grey to dark brown mycelia with whitish edges, measuring 2.5 – 3.1 cm in diameter after 7 days, with abundant production of conidia. The reverse side of the colony was black to dark brown in the centre, with irregular margins (Fig. 3E, 3F). Conidiophores were formed singly or in small groups, straight or flexuous, smooth, septate, cylindrical, geniculate above, pale or brown, and measured 124–193 × 5–10 μm. Conidia were straight or curved, ellipsoidal, dark olive-brown, smooth, measuring 37–91 × 11–30 μm and with 6–11 distosepta (Fig. 3G; 3H). These characteristics match the description provided by Sivanesan (1985*)* for this species.

Pathogenicity of B. gossypina on barley

Bipolaris gossypina UEM4658 and UEM4698 isolates were pathogenic to the Imperatriz cultivar. Small elongated brown spots, indicating tissue necrosis, were initially observed on the leaves. Most of these spots were surrounded by a faint and irregular yellow halo (Figs. 4A and 4B). Approximately 10 days after inoculation, elongated necrotic dark brown lesions with irregular borders measuring up to 1 cm in length were observed. Lesions may coalesce. These spots also showed a slight irregular chlorotic halo surrounding the necrotic lesion (Fig. 4C). In plants inoculated with *B. sorokiniana*, small, oval to oblong, brownish-black lesions with a yellow halo surrounding necrotic spots were initially observed on the leaves (Figs. 4D and 4E). As the lesions progressed, the necrotic lesions became slightly lighter brown than those caused by *B. gossypina* and presented a dark brown center. The lesions coalesced, causing larger patches of dead tissue on the leaves (Figs. 4F and 4G). Pathogenicity tests were conducted twice, and the fungi were consistently isolated from the inoculated plants.

Aggressiveness of B. sorokiniana isolates

All tested isolates were pathogenic to the ANA03 and Imperatriz cultivars, leading to symptoms of necrotic lesions with or without chlorotic margins, whereas the control did not show leaf spots.

The joint analysis of variance for severity data from the two experiments revealed significant effects of isolated factors and cultivars (< 0.001). However, the differences between the experiments were not significant; therefore, the data were pooled. The interaction between isolated factors and cultivars was also not significant (Online Resource 3). The isolates of *B. sorokiniana* showed significant differences in aggressiveness among cultivars (Table 2). The mean infection rate was higher in the Imperatriz cultivar (7.1) than in the ANA03 cultivar (6.3), indicating greater susceptibility of the former. The full range of IR variation among the isolates can be visualized in the descriptive analysis shown in Fig. 5.

Table 2

Mean infection responses[1] were compared for two barley cultivars inoculated with 16 isolates of *Bipolaris sorokiniana* collected in Paraná state, Brazil in 2021 and 2022

Isolate	Cultivar			
	Ana03		Imperatriz	
Control	0.0[2]	e	0.0	e
UEM4610	6.2	ad	7.3	ac
UEM4618	7.4	a	7.1	ac
UEM4625	6.3	ac	7.3	ac
UEM4631	6.1	ad	5.7	d
UEM4639	5.6	cd	7.1	ac
UEM4644	6.8	ac	7.2	ac
UEM4650	7.4	ab	6.9	ad
UEM4665	6.7	ac	8.1	a
UEM4674	5.5	cd	6.7	ad
UEM4687	5.1	d	7.4	ac
UEM4693	6.1	ad	7.9	ab
UEM4733	7.2	ab	7.6	ac
UEM4741	6.7	ac	6.6	bcd
UEM4749	6.4	ac	7.5	ac
UEM4751	5.6	cd	7.1	ac
UEM4756	6.0	bcd	6.3	cd
Mean	6.3	B	7.1	A
P > F	0.001			
CV%	19.9			

[1] Based on the visual scale of infection response developed by Fetch and Steffenson (1999), which uses a rating system of 1 to 9 to indicate the presence of necrosis and chlorosis, as well as the relative size of spot blotch lesions observed on the leaves of barley seedlings

[2] Mean followed by the same lowercase letter in each column or uppercase letter in each row within the corresponding mean, are not significantly different based on the LSD test (P = 0.05)

Discussion

DNA barcoding and phylogenetic analysis were used to identify 125 isolates of *Bipolaris* spp. associated with spot blotch disease in the barley-producing areas of Paraná State, Brazil, during the 2021 and 2022 growing seasons. *Bipolaris sorokiniana* was the prevalent species, accounting for 93.6% of the isolates, while *B. gossypina*, a species not previously found on barley, accounted for 6.4%. Species identification did not rely on a BLAST search in GenBank, as cautioned against by Bhunjun et al. (2020); instead, it was accomplished using phylogenetic analysis of the ITS region and gapdh and tef genes, as well as the combination of the three datasets (Marin-Felix et al. 2017; Bhunjun et al. (2020). The DNA barcoding approach outlined by Marin-Felix et al. (2017) and Bhunjun et al. (2020) allows the identification of up to 40 and 45 barcode species of *Bipolaris*, respectively.

This study represents the first report of *B. gossypina* as an emerging pathogen in barley. The leaf spot symptoms caused by *B. gossypina* were very similar to those caused by *B. sorokiniana*. Overall, it appears that *B. gossypina* causes less leaf damage than *B. sorokiniana*. Further surveys are necessary to better understand the extent of pathogenic variability of *B. gossypina* as well as the range of hosts and survival in different environments. *Bipolaris gossypina* was reported to occur in seed samples of *Gossypium* sp. from Kenya in 1985 (Sivanesan 1985). To date, no sexual morphs have been identified in this species (Bhunjun et al. 2020). Additionally, *B. zeicola* found on barley seeds in Argentina was shown to be pathogenic to barley, causing leaf spots (Cipollone et al. 2020). *Bipolaris zeicola* (Syn.: *Cochliobolus carbonum* R.R. Nelson) is a well-known pathogen of maize (White 1999). Other *Bipolaris* species reported on barley include *B. spicifera, B. victoriae, B. australiensis, B. cynodontis, B. gigantea, B. hawaiiensis*, and *B. setariae* (Farr and Rossman 2023). However, no information is available regarding the symptomatology and damage caused by these species in barley. Moreover, among these species, only *B. cynodontis, B. setariae*, and *B. sorokiniana* have been accepted by Marin-Felix et al. (2017) and Bhunjun et al. (2020).

The origin of the *B. gossypina* strains that infect barley remains unclear. The vast Brazilian cotton cultivation area, reaching approximately 1.6 million hectares, is at least 400–500 km from the barley area in the tropical region of the country. Furthermore, *B. gossypina* has not been previously reported in cotton in Brazil. It is reasonable to hypothesize that this species could have transitioned from native plant species to barley. Further studies including a larger number of geographically representative isolates may deepen our understanding of the impact of this and other *Bipolaris* species on barley production in subtropical southern Brazil. Certainly, the expansion of barley production in the central region of Brazil in the tropical highlands of the Cerrado region, the Brazilian savannah, requires attention.

This study did not reveal high diversity of aggressiveness among the *B. sorokiniana* isolates affecting barley in Brazil. The infection rates ranged from 5.1 to 7.4 in the cultivar ANA03 and 5.7 to 8.1 in the cultivar Imperatriz, on a 1 to 9 scale. In contrast, Ghazvini and Tekauz (2007), who used a gene-for-gene model to evaluate the reaction of isolates with differential lines using the same infection rate scale as this study but applying the virulence concept, found high diversity of virulence in Canada, with infection rates ranging from 2 to 8. To differentiate lines, the study considered infection rates < 4.4 and > 4.5 as resistant and susceptible reactions, respectively. The infection rates in the present study were higher than 4.5, indicating that both cultivars were susceptible. Therefore, the term aggressiveness was preferred over virulence to describe the interaction of *B. sorokiniana* isolates with barley in this study, as used by Gamba et al. (2020). To the best of our understanding, it expresses the quantitative reactions found in this study more clearly.

The interaction between the isolates and cultivars was not significant in this study. The study was conducted with only two cultivars, based on previous information that ANA03 presented greater field resistance to spot blotch than Imperatriz. Previous studies have shown that resistance to spot blotch in barley is generally a quantitative trait, indicating that it is controlled by multiple genes and is explained by the lack of interaction between isolates and

cultivars. However, there is evidence suggesting that qualitative resistance, explained by the gene-for-gene model, may play a role in some interactions (Ghazvini and Tekauz 2007; 2008). A study by Gamba et al. (2020) also revealed some degree of isolate-specific interaction, indicating the presence of some level of qualitative resistance, although resistance was predominantly quantitative. Unlike in barley, differential interactions between *B. sorokiniana* and wheat genotypes have not yet been reported (Ghazvini, 2018). Knowing the interaction between isolates and host plant genotypes is essential for selecting pathogen isolates for use as inocula in screening barley genotypes for resistance to spot blotch. Therefore, the information generated in this study will contribute to the search for barley germplasms with better genetic resistance to spot blotch.

Declarations

Data availability The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest The authors declare no competing interests.

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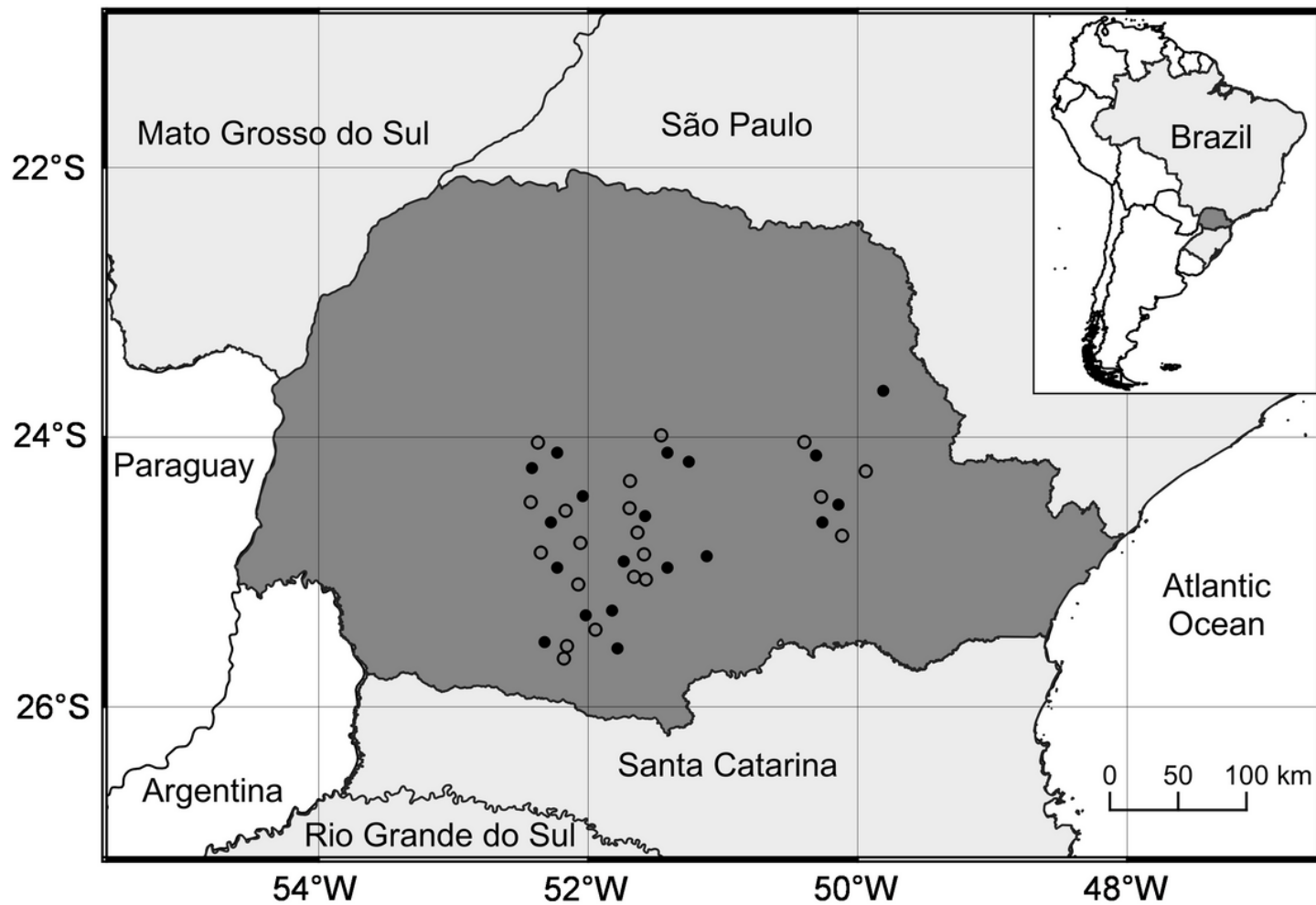
Figures

Figure 1

Map of Paraná State (dark grey) in Brazil (light grey), indicating the locations where samples of *Bipolaris sorokiniana* and *B. gossypina* were collected in 2021 (empty circles) and 2022 (full circles).
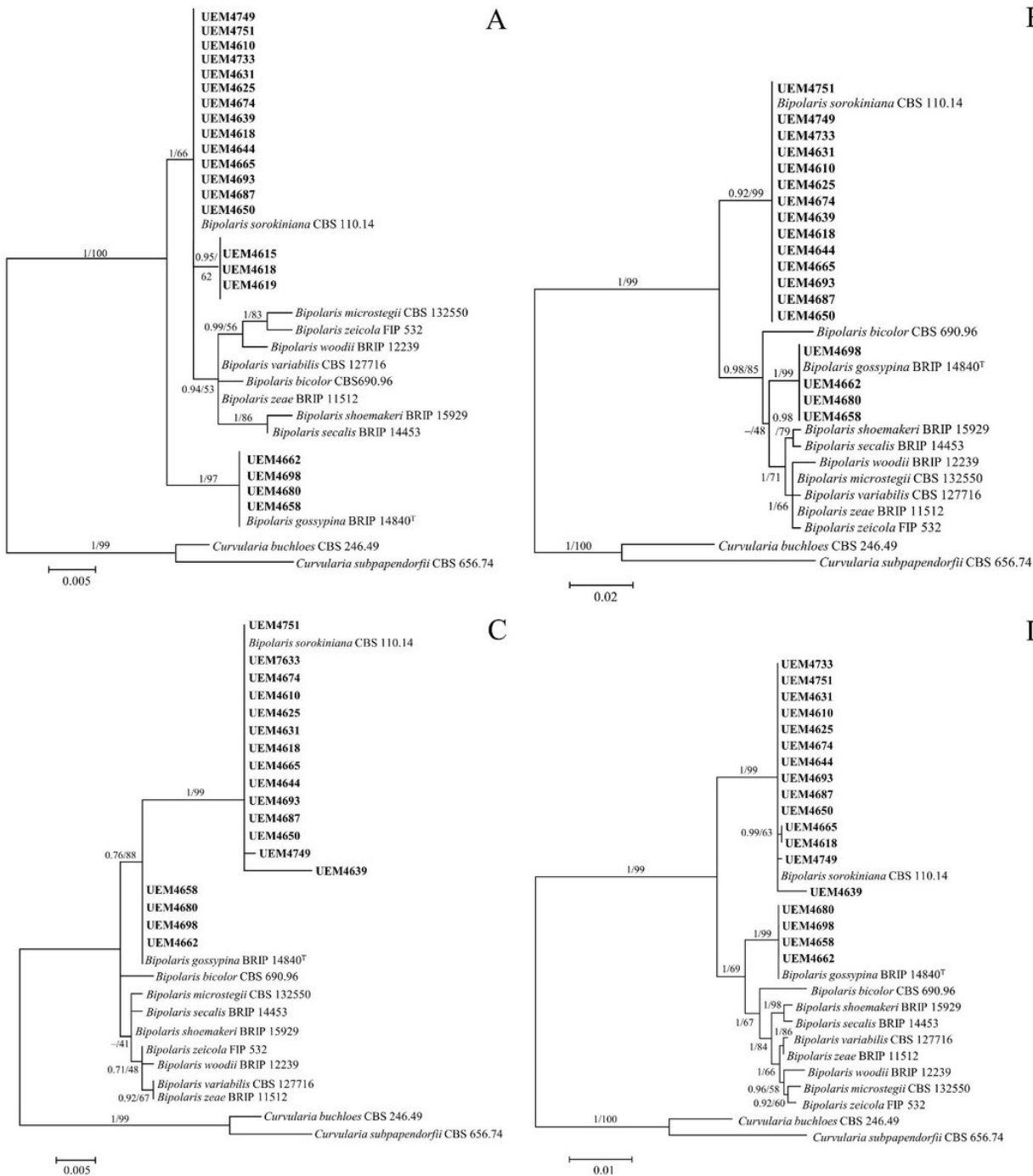
Figure 2

Phylogenetic trees from Bayesian inference based on ITS (A), *gapdh* (B), *tef1* (C), and concatenated datasets (D) of the *Bipolaris* species. Bayesian posterior probability (B-PP) values >50% and bootstrap values obtained using the maximum likelihood method (ML-BS) >50% are shown at the nodes (B-PP/ML-BS). The tree is rooted with *Curvularia buchloes* CBS 246.49 and *C. subpapendorfii* CBS 656.74. The isolates obtained in this study are shown in bold font. [T] ex-type
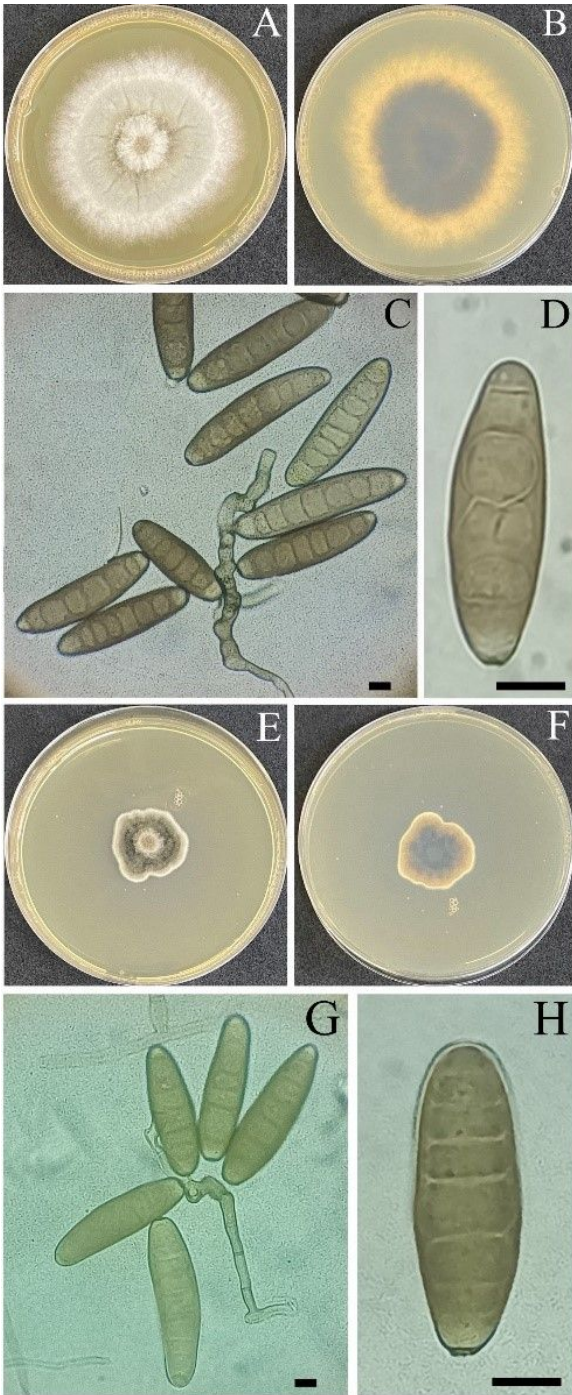
Figure 3

Morphological characteristics of *Bipolaris gossypina* UEM4658 (A-D) and *B. sorokiniana* UEM4610 (E-H). *Bipolaris gossypina* 5-day-old colony on PDA (A) and V8 (B) media, conidiophore and conidia (C, D). *Bipolaris sorokiniana* 7-day-old colony on PDA (E) and V8-(F) media, conidiophore and conidia (G, H). Scale bars: 10 μm.
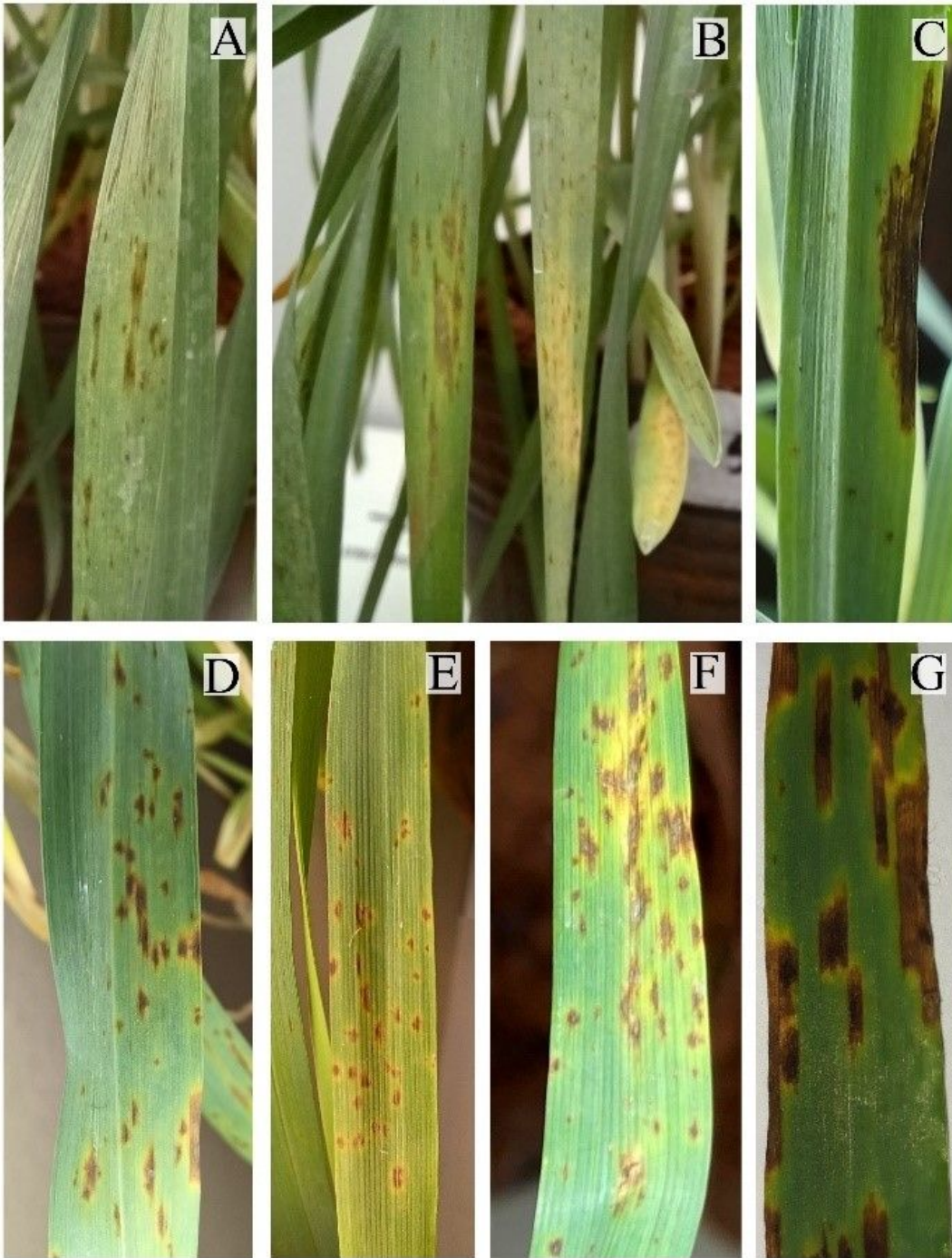
Figure 4

Leaf spot symptoms in barley caused by the isolate *Bipolaris gossypina* UEM4658 at five days (A, B) and 10 days (C) after inoculation. Spot blotch symptoms caused by the isolate *B. sorokiniana* UEM4610 at five days (D, E) and 10 days (F, G) after inoculation
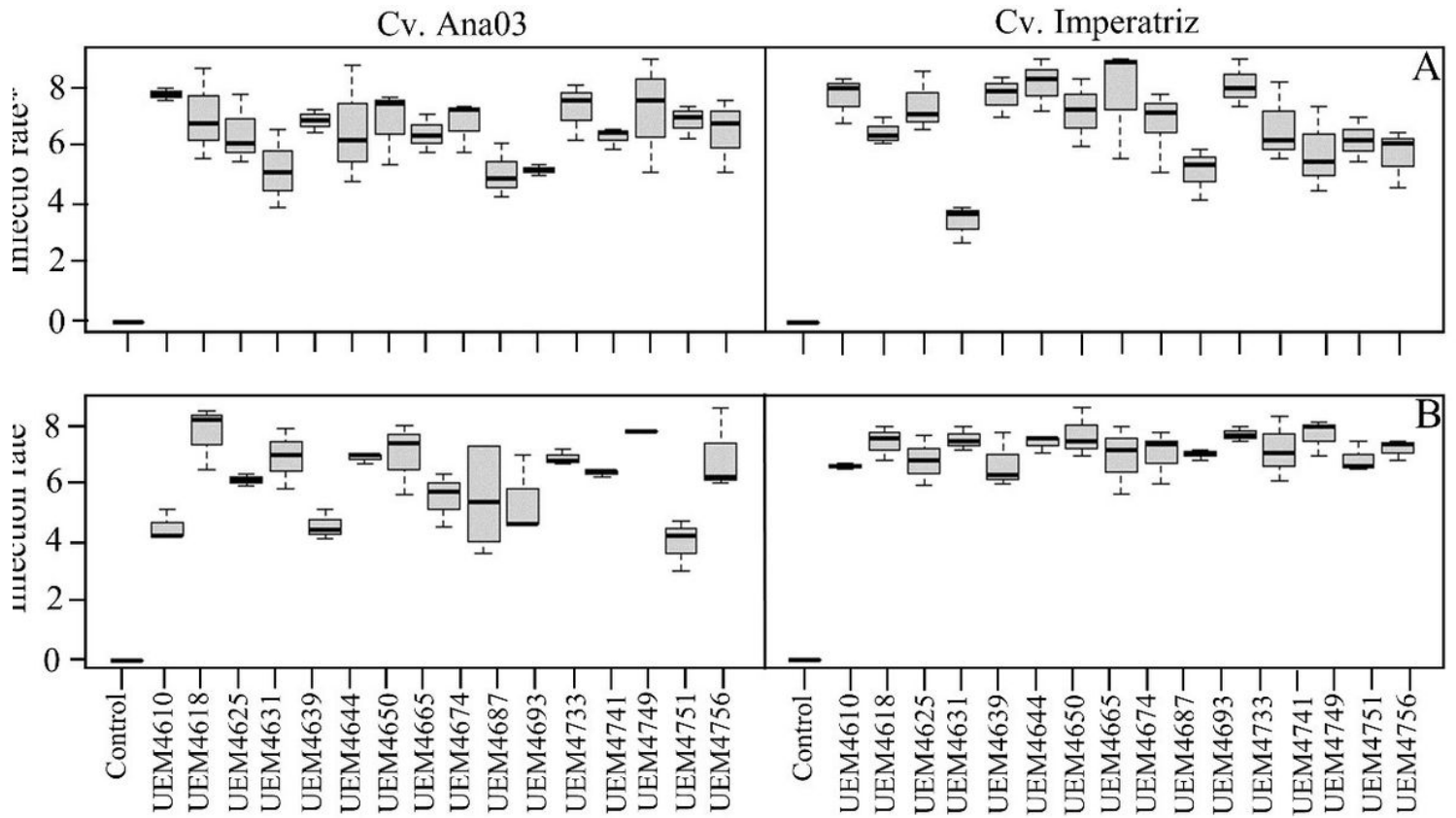
Figure 5

Box plot of the distribution of mean aggressiveness scores of *Bipolaris sorokiniana* isolates in barley cultivars ANA03 and Imperatriz in experiments 1 (A) and 2 (B). The solid line within the box refers to the mean, and the box shows the 25th and 75th percentiles of the data. The vertical bars extending from the boxes representthe 10% and 90% percentiles, respectively. *1-9 infection rating scale developed by Fetch and Steffenson (1999).

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