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Ilyonectria Species Associated With Tree Decline in *Pinus taeda* in Brazil

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Keywords: needle chlorosis | phylogeny | soil fungi

ABSTRACT

Characteristic symptoms of decline were observed in 10-year-old *Pinus taeda* (loblolly pine) trees in plantations located in the state of Santa Catarina, in the humid subtropical south of Brazil. Aboveground, we observed needle chlorosis, followed by drying and shortening of needles, formation of tufts on branch tips, and death of the canopy at a more advanced stage. In the root system, there was a reduction in the volume of secondary roots, the absence of ectomycorrhizae, and some external necrotic lesions. This study aimed to identify the pathogenic fungi associated with the decline. Roots and soil from symptomatic trees were collected to isolate pathogenic fungi. Molecular characterisation of the isolates was carried out by sequencing the ITS region and partial HIS3 and TEF1 genes; morphological characterisation of conidiophores and conidia was also conducted. Seven isolates were identified, belonging to the species *Ilyonectria leucospermi* ($n = 2$), *I. protearum* ($n = 2$), *I. robusta* ($n = 2$), and *I. vredenhoekensis* ($n = 1$). Koch's postulates were fulfilled for the pathogenic characterisation of the isolates on *P. taeda* seedlings. This study is the first to report these pathogens causing disease in *P. taeda* worldwide, and it demonstrated their association with the decline of pine trees in Brazil.

1 | Introduction

Brazil is a major global leader in the export of cellulose products and in the forest productivity of fast-growing species, such as eucalyptus and pine (IBÁ 2024). The genus *Pinus* (mainly represented by *P. taeda* L. and *P. elliottii var. elliottii* Engelm.) constitutes the second largest forest base in Brazilian forestry, with approximately 1.9 million ha planted. These are concentrated in the states of the southern region of Brazil, with Paraná as the largest producer and Santa Catarina in second place nationally (IBÁ 2024). Pine trees have light-coloured wood and long fibres, allowing their wood products to be used in various sectors of the forestry industry, such as in the production of cellulose, high-tensile strength paper and laminates for the furniture sector (Braga et al. 2020).

The main diseases affecting pine trees in Brazil are Armillaria root rot, caused by *Armillaria* sp., and shoot dieback, caused by *Diplodia sapinea* (*Sphaeropsis sapinea*). These diseases lead to rotting of the bark, wood and crown of the plant, as well as a dried-out canopy, ultimately resulting in the death of the plant (Auer and dos Santos 2016). Cylindrocladium root rot was also

observed in nurseries and trees (Auer and dos Santos 2016). However, these diseases have occurred infrequently (Auer and dos Santos 2016) and are not considered significant barriers to cultivation.

Since 2014, part of the *P. taeda* producing regions in southern Brazil, notably in the states of Santa Catarina and Paraná, have systematically presented a complex symptom. The first symptoms are observed in the aerial part of the trees, including yellowing and chlorosis of needles at various levels, reduction in size and formation of tufts on branches and branch tips. Symptoms begin to be seen in plantations that are 10 years old and, in extreme situations, death occurs in trees over 16 years old. Examination of the root system of symptomatic trees revealed the presence of thicker roots with dark lesions, and a reduction in volume or absence of living fine roots and active ectomycorrhizae. This condition, known as 'Decline of Pine Trees' (DPT), leads to widespread decline in trees.

This disease has been given different names depending on the most visible symptom, one of which is chlorosis (Auer et al. 2019; Motta et al. 2024). Brumat et al. (2023) associated a decline of *P. taeda* trees with *Phytophthora macrochlamydospora*. Thus, it appears that the decline or chlorosis of *P. taeda* trees in Brazil has a complex aetiology, suggesting a biotic–abiotic interaction. Similar anomalies in North America have been observed in *Pinus echinata*, *P. taeda*, *P. elliottii* and *P. palustris*, known as Southern Pine Decline (Coyle et al. 2015) or Little Leaf Disease (Campbell and Coyle 2016).

Preliminary isolations made from samples of fine roots showing necrosis and rhizosphere soil of pine trees with decline symptoms, obtained in plantations of Southern Brazil, revealed that fungal cultures were consistently obtained with abundant production of conidia in free conidiophores, characteristic of species belonging to the genus *Ilyonectria*.

There are reports of *Ilyonectria* causing root rot associated with decline diseases in forestry and woody agricultural species, in particular the decline of grapevines in Brazil (Cabral, Groenewald, et al. 2012; Dos Santos et al. 2014; Haavik et al. 2015), Canada (Úrbez-Torres et al. 2014), and Spain (Martínez-Diz et al. 2018); death of loquat trees in Spain (Agustí-Brisach et al. 2016); forest nurseries in Spain (Mora-Sala et al. 2018); *Eucalyptus smithii* trees in Uruguay (De Benedetti et al. 2024); kiwi in Turkey (Erper et al. 2013); and apple in Italy (Manici et al. 2018). Therefore, this study aimed to identify the species of *Ilyonectria* obtained from symptomatic *P. taeda* plants in Southern Brazil through pathogenicity tests, molecular analysis, and morphological characteristics.

2 | Material and Methods

2.1 | Root Sampling and Collection

Between the years 2019 and 2020 samples of fine roots were collected from symptomatic pine trees in four plantation areas located in the state of Santa Catarina. The plantation located in the municipality of Calmon (26°42′ 43.03″ S; 50°51′ 2.57″ W) consisted of 11-year-old trees, and the root and soil sampling was carried out in the autumn (10 June 2019). The area located in Correia Pinto (27°32′ 44.04″ S; 50°31′ 30.50″ W) contained 14-year-old trees, and sampling was conducted in the spring (26 September 2019). The tree plantations in the municipalities of Lages (27°44′ 26.78″ S; 50°29′ 26.82″ W) and São José do Cerrito (27°39′46″ S, 50°34′48″ W) were 19 and 14 years old, respectively, and both were sampled in the summer (10 March 2020). Each area consisted of 1 ha, and within the plot, 15 trees were selected with characteristic symptoms of needles with chlorosis and reduced size, few fine roots, and absence of ectomycorrhizae. For each tree, samples of four thin roots (up to 0.5 cm) were collected from approximately 1 m from the tree, at opposing cardinal directions. These were then combined to constitute a composite sample of 200 g of roots. The 60 composite samples were stored in plastic bags and coolers with ice and taken to the Forest Pathology Laboratory at Embrapa Florestas, Colombo, Paraná, Brazil. In each plot, two trees were cut down and transverse and longitudinal cuts were made in the stem to observe the presence or absence of tissue darkening.

2.2 | Isolation and Selection of *Ilyonectria* Isolates

Root samples from symptomatic pine trees were washed in running water and placed on filter paper to remove excess water. Then, the symptomatic regions of the roots were separated for fungal isolation. For each composite sample, ten petri dishes containing five root fragments per dish were used. Indirect isolation was carried out by first disinfecting in the transition region between healthy and symptomatic material (30 s in 70% alcohol solution +90 s in 0.5% sodium hypochlorite solution + three washes in sterilised water). The fragments were transferred to Petri dishes containing a selective medium of malt extract and antibiotics used by Eckhardt et al. (2007) and Zanzot et al. (2010) with the concentrations of streptomycin sulphate and cycloheximide adjusted to 0.5 ppm and 40 ppm, respectively. The plates were incubated in a growth chamber at 24°C in the dark for 7 days. After incubation, a visual evaluation of the colonies and microscopic observations at 400× magnification (Zeiss Axioscope microscope) were performed, and the isolates were subcultured on Petri dishes containing potato dextrose agar (PDA) medium.

A total of seven isolates, two from Calmon, two from Correia Pinto, two from Lages and one from São José do Cerrito, were obtained and preserved by the Castellani method in glass vials (penicillin vials) with sterile distilled water, kept in the Collection of Forest Fungi and Oomycetes at the Forest Pathology Laboratory at Embrapa Florestas.

For the identification and characterisation of the species, the isolates were grouped based on similarities in colony pigmentation and asexual structures. Monosporic cultures of seven isolates were obtained according to the methodology of Jarek et al. (2018), with two isolates selected from each of the areas of Calmon (strain number IIIA3SP1 and IIIM4RP1), Correia Pinto (strain number VA1SPF6 and VA3RPF7) and Lages (strain number VIIB3RF1 and VIIB4RF3), and one isolate (strain number VIIIA1RF1) selected from São José do Cerrito. Molecular and morphological characterisation, and pathogenicity testing were performed for this group of isolates.

2.3 | Molecular Characterisation of *Ilyonectria* Isolates

DNA amplification of the ITS region and the partial HIS3 and TEF1 genes was performed for the seven isolates. DNA extraction was performed using 150 mg of mycelia from the isolates grown in Petri dishes containing PDA medium for 14 days. DNA was extracted using the Wizard Magnetic DNA Purification System kit (Promega, Madison, USA), according to the manufacturer's recommendations. DNA was quantified and assessed for purity using a Nanodrop2000 (Thermo Fisher Scientific, Waltham, MA).

Genomic DNA samples were amplified in a Veriti thermocycler (Thermo Fisher Scientific) in a reaction mixture containing 20 ng of purified genomic DNA. For the amplification of the ITS region, the primers ITS1 and ITS4 were used, and the cycling conditions were as follows: 32 cycles at 96°C for 30 s, 58°C for 45 s, and 72°C for 45 s (White et al. 1990). For the HIS3 gene, primers CYLH3F and CYLH3R were used, and the cycling conditions were as follows: 96°C for 5 min; 30 cycles at 96°C for 30 s, 52°C for 30 s, and 72°C for 60 s; and a final extension step at 72°C for 5 min (Crous et al. 2004). For the TEF1 gene, the primers EF1–1018F and EF1–1620R were used, with the following cycling conditions: 35 cycles at 96°C for 30 s, 56°C for 1 min, and 72°C for 45 s (Stielow et al. 2015) The PCR products were enzymatically purified with ExoI/SAP (Thermo Fisher Scientific), following the manufacturer's recommendation.

Sample sequencing was performed on a Genetic Analyzer 3500xL (Thermo Fisher Scientific) using 50-cm capillaries with Pop7 polymer (Thermo Fisher Scientific), according to the manufacturer's instructions. The electrophoretograms generated were converted into base sequences using the Sequencing Analysis v5.4 programme (Thermo Fisher Scientific).

Electrophoretograms were verified in the BioEdit 7.2.5 software, and the forward and reverse sequences were aligned to generate consensus sequences. The consensus sequences were aligned using ClustalW in MEGA 11, and phylogenetic trees were inferred from this file. The sequences obtained were compared with accessions deposited in GenBank using 'Nucleotide BLAST' on the NCBI server (www.ncbi.nlm.nih.gov) for evolutionary analysis.

Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) on the CIPRES web portal (Miller et al. 2011). Bayesian inference was performed using MrBayes in the XSEDE tool (Ronquist et al. 2012) using the Markov chain Monte Carlo (MCMC) algorithm. The analysis was performed using GTR + I + G models, and 10 million generations were run for the dataset. The run was stopped automatically when the average standard deviation of the divided frequencies was below 0.01. Trees were sampled every 1000 generations, and the burn-in was 25%. The remaining trees were used to calculate posterior probabilities (PP). The maximum likelihood analysis was performed using the RAxML-HPC BlackBox tool (Stamatakis 2014). One thousand non-parametric bootstrap iterations were employed using the generalised time-reversible (GTR) model and a discrete gamma distribution. The sequence of *Campylocarpon fasciculare* (CBS 112613) was used as an outgroup. The sequences used were obtained from the GenBank database and are listed in Table S1.

2.4 | Morphological Characterisation of *Ilyonectria* Isolates

Morphological characterisation was conducted on the same seven isolates that were previously characterised at the molecular level. To produce asexual structures, the microculture technique (Alfenas and Mafia 2007) was used with oatmeal-agar (OA) medium (60 g of oat flour, 15 g of agar in 1000 mL of sterilised ultra-purified water) and synthetic nutrient-poor agar (SNA) medium (10 g agar, 500 mL ultra-purified water, 0.50 g monopotassium phosphate, 0.50 g potassium nitrate, 0.25 g magnesium sulphate heptahydrate, 0.25 g potassium chloride, 0.10 g of glucose, 0.10 g of sucrose) (Cabral, Groenewald, et al. 2012).

A cube of the culture medium, measuring approximately 1 cm^2, was placed on a sterilised glass slide inside a petri dish. The fungus was transferred to four sides of the culture medium block, and a sterilised coverslip was placed over the culture medium. The material was placed in a moist chamber and incubated at 24°C with a 12-h photoperiod for 7 days. Slides were prepared to view and measure the structures. For all evaluations, 30 structures were examined using a ZEISS Axioscope microscope at 400× magnification.

Sporodochia were produced using a clove leaf-agar medium with 2% agar and five clove leaf fragments (Cabral, Groenewald, et al. 2012). Pure colonies were incubated at 24°C with a photoperiod of 12 h of light for 45 days. Slides were prepared for viewing and measuring the structures. For all evaluations, 30 structures were measured using a ZEISS Axioscope microscope at 400× magnification. The isolates included in this study were characterised based on the morphology of conidiophores, macroconidia, microconidia, presence of sporodochia and chlamydospores (Figure S1).

2.5 | Pathogenicity Test of *Ilyonectria* Isolates

The pathogenicity of the seven selected isolates was assessed using the pine seedling inoculation method with colonised sorghum grains, following the protocols described by Jarek et al. (2018), Lombard et al. (2013) and Vargas (2020). Sorghum grains were placed in 500 mL glass jars with ultra-purified water (80 g of grains/50 mL of water) and then autoclaved at 121°C, 101 kPa, for 30 min. After autoclaving, the jars were shaken to loosen the grains and were left to cool.

The fungal isolates were grown on PDA medium in the dark at 24°C for 14 days. Ten discs of mycelia from each isolate, 5 mm in diameter, were transferred to two jars containing sorghum grains, which were subsequently incubated at 25°C for 10 days and shaken daily to homogenise the colonisation of the grains by the fungi.

The sterilised plant growth substrate (sphagnum peat, expanded vermiculite, dolomite limestone, agricultural gypsum and 04-14-08 NPK fertiliser) was homogenised with

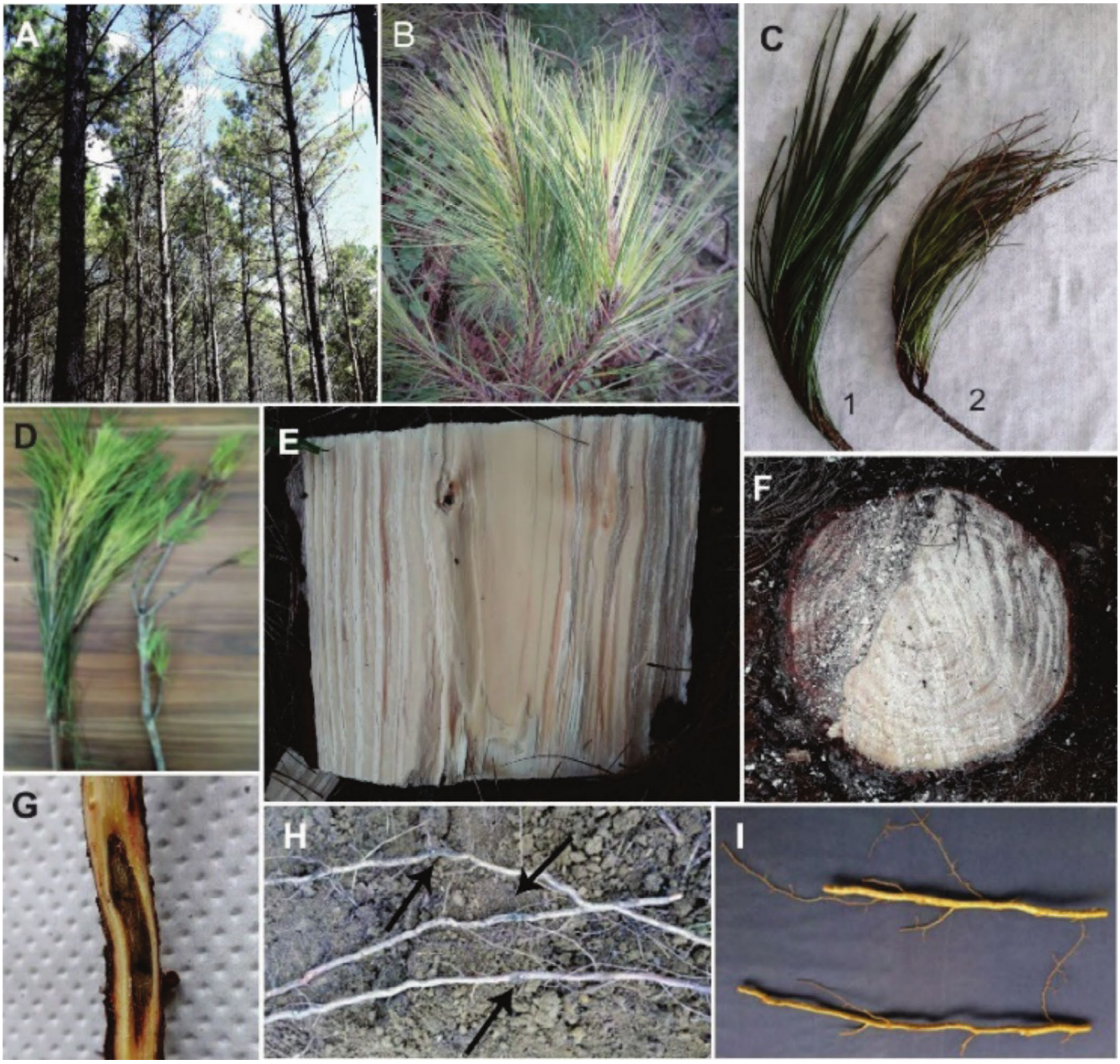

FIGURE 1 | Symptoms of *Pinus taeda* decline of trees in Brazil: (A)—trees in pine plantations showing needle chlorosis, reduced development, and death; (B)—chlorosis in pine needles; (C)—(1) healthy needles and (2) needles with reduced length and showing dryness from tip to base; (D)—needle chlorosis and formation of tufts on branch tips; (E)—longitudinal section of symptomatic pine tree; (F)—cross-section of symptomatic pine tree; (G)—root with internal necrosis; (H)—roots with external necrotic lesions; (I)—small volume of fine roots and absence of ectomycorrhizae.

the inoculum (40 g of colonised grains/L of substrate). Subsequently, 6-month-old pine seedlings with a 3- to 4-mm stem diameter were transplanted into previously homogenised substrate in 220-cm^3 tubes.

For the pathogenicity test, an experiment was conducted in a completely randomised design with eight treatments and four replicates. The seedlings were obtained from a commercial nursery and propagated from seeds. A total of 96 seedlings were used, with three seedlings/replicate inoculated with each of the seven *Ilyonectria* isolates and one control group (sterilised sorghum grains without fungus). About three to five fine roots of the seedlings were slightly injured by scraping with a knife at the time of transplanting. After inoculation, all seedlings were placed in a plant growth chamber (Environ Plant, Brazil) at 25°C ± 2°C under a 12-h photoperiod for 180 days, with daily irrigation. At 180 days after inoculation (dpi), the seedlings were evaluated through visual analysis of root and stem darkening, needle chlorosis, and plant death. Following the assessment, fungal re-isolation from the roots was carried out to fulfil Koch's postulates.

The incidence of dead plants for each isolate was assessed, as well as the incidence of plants showing symptoms of root and stem darkening and needle chlorosis. The data were submitted to the Shapiro–Wilk normality and Bartlett's homogeneity of variance tests. Analysis of variance (ANOVA) was conducted with the test of comparison of means (Scott-Knott test) at the

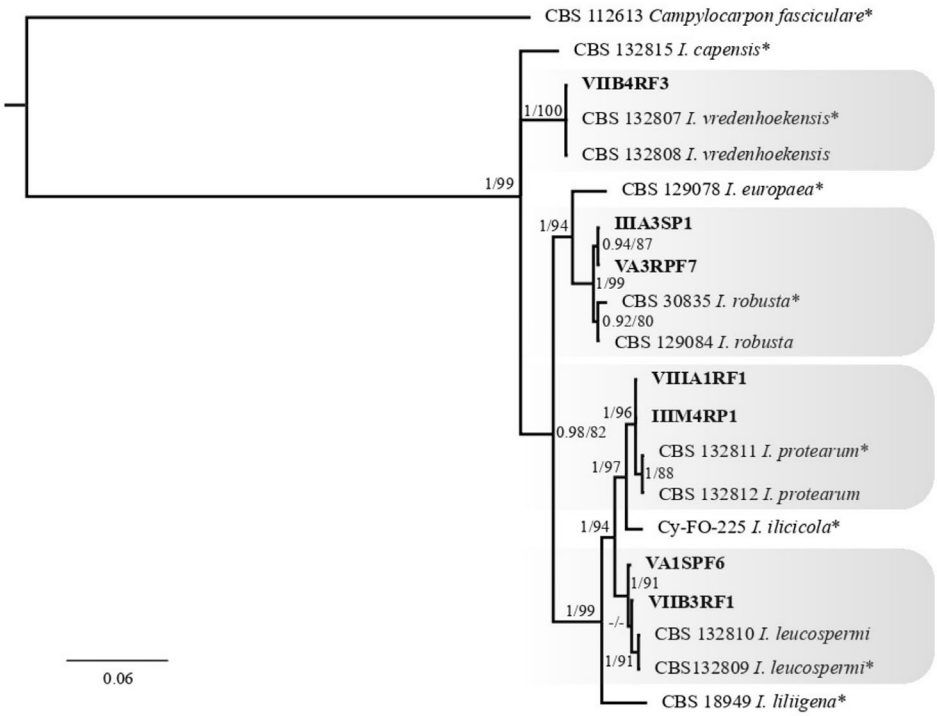

FIGURE 2 | Phylogram from Bayesian inference of the ITS region and tef1 and his3 genes of *Ilyonectria* species associated with the decline of *Pinus taeda* trees in Southern Brazil. Codes in bold refer to isolates used in this study, and other codes refer to sequences from GenBank. Values of Bayesian posterior probabilities (BPP) and bootstrap values of maximum likelihood support values (MLBS) > 70% are indicated at the nodes (BPP/MLBs). The scale bar represents the expected number of changes per location. The species *Campylocarpon fasciculare* (CBS112613) was used as an outgroup. *type isolate.

level of significance of 5%. Statistical analysis was performed using the R software (R Core Team 2025).

3 | Results

3.1 | Characterisation of Pine Decline Symptoms

The symptoms observed in the canopies of symptomatic *P. taeda* trees began with needle chlorosis, followed by drying and abscission. Reduced needle growth and tuft formation on branches and branch tips were also observed. In the most severe cases, death of the tree canopy was observed (Figure 1A–D).

In a more in-depth investigation, a transverse and longitudinal section was made of the stems of some symptomatic trees, and no darkening of the internal tissues was observed (Figure 1E,F). The root systems of symptomatic trees were inspected, and a significant decrease in the volume of fine roots was observed. In some roots, up to approximately 2 cm in diameter, darkening was observed when a longitudinal cut was carried out (Figure 1G–I).

3.2 | Molecular Characterisation of *Ilyonectria* Isolates

A BLAST search of the GenBank database revealed that the sequence fragments of the ITS region and TEF1 and HIS3 genes of the isolates from this study were 97%–100% similar to isolates used as references for the species *I. vredehoekensis* L. Lombard & Crous, *I. protearum* L. Lombard & Crous, *I. leucospermi* L. Lombard & Crous (Lombard et al. 2013) and *I. robusta* (A.A. Hildebr.) A. Cabral & Crous (Cabral, Groenewald, et al. 2012). The phylogenetic tree inferred from concatenated datasets from the ITS region and TEF1 and HIS3 genes revealed that isolate VHB4RF3 grouped into the clade of *I. vredehoekensis*, while isolates VHB3RF1 and VA1SPF6 clustered in the clade of *I. leucospermi*. Isolates IIIM4PPI and VIIIA1RF1 grouped with *I. protearum*, while VA3RPF7 and IIIA3SP1 clustered with *I. robusta* (Figure 2). These clades were statistically supported by Bayesian probability values of 1.0 and bootstrap values of 91% or higher. These clades encompass the nomenclatural types (ex-type) of the species. The clades of all species contained subclades, indicating the genetic variability within each species.

3.3 | Morphological Characterisation of *Ilyonectria* Isolates

Ilyonectria leucospermi isolates produced solitary conidiophores with macroconidia and microconidia, which had a visible hilum. Sporodochia were not observed. The macroconidia were septate (1–3 septa), straight, and had cylindrical ends; in some cases, a hilum at the tip of the conidium was visible. The microconidia were oval and fusiform, aseptate, or had one septum. The aseptate microconidia measured 7–13 × 6–3 μm, and the septate microconidia measured 21–13 × 6–4 μm (Table 1). Abundant production of chlamydospores was observed across all evaluated culture media. Chlamydospores were formed both terminally and intercalarily within the hyphae and appeared in

TABLE 1 | Morphological characteristics of *Ilyonectria* species from this study and reference species.

Characteristics	*I. robusta* (present study)	*I. robusta* (Cabral, Rego, et al. 2012; Cabral, Groenewald, et al. 2012)	*I. leucospermi* (present study)	*I. leucospermi* (Lombard et al. 2013)	*I. protearum* (present study)	*I. protearum* (Lombard et al. 2013)	*I. vredehoekensis* (present study)	*I. vredehoekensis* (Lombard et al. 2013)
Conidiophores	Solitary or aggregated	Solitary or aggregated	Solitary	Solitary	Solitary	Solitary	Solitary	Solitary
Sporodochia	Present	Present	NO	NO	Present	Present	Present	Present
Conidia	Macroconidia and microconidia	Macroconidia and microconidia	Macroconidia and microconidia	Macroconidia and microconidia	Macroconidia and microconidia	Macroconidia and microconidia	Macroconidia and microconidia	Macroconidia and microconidia
Hilum	NO*	NO	Observed	Observed	Observed	Observed	Observed	Observed
Macroconidium shape	Straight with rounded ends, slightly curved or distorted, narrowing towards the tip of the conidium	Straight with rounded ends, slightly curved or distorted, narrowing towards the tip of the conidium	Straight with rounded ends	Straight with rounded ends	Straight with rounded ends	Straight with rounded ends	Straight with rounded ends	Straight with rounded ends
Septate macroconidium dimensions (μm)	28-(24)-22×7-(6)-6**	25-(24)-23×7-(7)-6	30-(24)-21×7–6-5	26-(23)-20×7-(6)-5	29-(25)-20×7-(6)-6	27-(24)-21×7-(6)-5	29-(25)-22×8-(7)-5	28-(26)-24×7-(6)-5
2-Septa macroconidium dimensions (μm)	34-(29)-25×8–7-5	28-(27)-26×7-(7)-7	39-(33)-29×7-(6)-6	37-(34)-31×7-(7)-5	32-(29)-23×8-(6)-5	34-(30)-26×8-(7)-6	38-(30)-26×8-(7)-6	30-(28)-26×8-(7)-6
3-Septa macroconidium dimensions (μm)	40-(34)-29×8-(7)-6	35-(34)-32×8-(7)-7	43-(39)-31×8-(7)-6	37-(34)-31×8-(7)-6	38-(34)-28×8-(7)-6	38-(35)-32×9-(8)-7	40-(34)-30×8-(7)-6	34-(32)-30×7-(6)-5
Microconidium shape	Ellipsoid and ovoid	Ellipsoid, ovoid, sub cylindrical	Oval and fusiform	Oval, fusiform, globose	Oval and fusiform	Oval, ovoid, fusiform	Ellipsoidal and oval	Fusiform, ellipsoidal, and ovoid

(Continues)

TABLE 1 | (Continued)

Characteristics	*I. robusta* (present study)	*I. robusta* (Cabral, Rego, et al. 2012; Cabral, Groenewald, et al. 2012)	*I. leucospermi* (present study)	*I. leucospermi* (Lombard et al. 2013)	*I. protearum* (present study)	*I. protearum* (Lombard et al. 2013)	*I. vredehoekensis* (present study)	*I. vredehoekensis* (Lombard et al. 2013)
Aseptate microconidium dimensions (μm)	11-(9)-7×4-(4)-3	9-(9)-8×4-(4)-4	13-(10)-7×6-(5)-3	13-(11)-9×5-(4)-3	15-(12)-10×5-(5)-4	14-(12)-10×5-(4)-3	12-(9)-6×6-(4)-3	8-(7)-6×4-(3)-3
Septate microconidium dimensions (μm)	20-(15)-12×6-(5)-4	15-(14)-14×5-(5)-5	21-(17)-13×6-(5)-4	21-(18)-15×6-(5)-4	19-(16)-14×6-(5)-4	18-(17)-14×6-(5)-4	19-(15)-12×7-(5)-4	17-(15)-12×6-(3)-4
Chlamidospore	Present	Present	Present	Present	Presente	Present	Present	Present
Diameter (μm)	15-(12)-09	14-(NO)-7	20-(15)-11	20-(NO)-8	19-(15)-12	18-(NO)-11	14-(10)-8	14-(NO)-8

*NO: Not observed.
**Maximum-(average)-minimum.

chains. These structures were hyaline, turning golden brown upon maturity, globular with a thick wall, and had diameters ranging from 20 to 11 μm (Table 1).

Ilyonectria protearum isolates had solitary conidiophores, and small cream-coloured sporodochia were observed in isolates of the species. There was abundant production of septate macroconidia (1–3 septa), straight with rounded ends, and a hilum at the tip of the conidia was observed. The dimensions of the macroconidia varied according to the number of septa: macroconidia with 1 septum measuring 29–20×7–6 μm; macroconidia with 2 septa measuring 32–23×8–5 μm; and macroconidia with 3 septa measuring 38–28×8–6 μm. The microconidia were oval and fusiform, aseptate (15–10×5–4 μm), and septate (19–14×6–4 μm), and the chlamydospores were globose to subglobose, thick-walled, intercalary, terminal and in chains (Table 1).

Ilyonectria robusta had mostly solitary conidiophores, but complex conidiophores and greenish-to-dark, large and abundant sporodochia were observed. Hyaline macroconidia and microconidia without a visible hilum were formed, in which the predominant formation of macroconidia occurred. Macroconidia of 1–3 septa were observed, straight with rounded ends, and some slightly curved and narrowed at the tip. The microconidia formed were mostly ellipsoid in shape, with some ovoid and subglobose, aseptate, or with a septum. Abundant production of terminal or intercalary chlamydospores was observed. The chlamydospores were globose to subglobose in shape, with thick walls, ranging from 14 to 17 μm in diameter, and hyaline, becoming golden brown at maturity (Table 1).

Ilyonectria vredehoekensis isolates formed solitary conidiophores and sporodochia, similar to most species in this study. Macroconidia of up to three septa were observed, straight with rounded ends, and the hilum was visible. The microconidia were ellipsoidal and mostly oval, with sizes ranging from 12 to 6×6–3 μm, aseptate or septate, with sizes ranging from 19 to 12×7–4 μm. Globose and subglobose chlamydospores were abundantly formed, with thick walls and a golden-brown colour when mature (Table 1).

3.4 | Pathogenicity Test of *Ilyonectria* Isolates

All isolates of *Ilyonectria* species were pathogenic to *P. taeda* and caused root and stem darkening symptoms and needle chlorosis, followed by seedling death (Figure 3) when evaluated 180 days after inoculation. The incidence of plants with root and stem darkening, needle chlorosis, and plant mortality was evaluated for all 12 seedlings assessed per isolate and is presented in Table 2. All seedlings inoculated with the seven isolates of the pathogens exhibited some symptoms of the disease, with the incidence of dead plants for the different isolates ranging from 8.3% to 83.3%. The isolates VIIB3RF1, VIIIA1RF1, IIIA3SP1, VA3RPF7 and VIIB4RF3 exhibited the highest aggressiveness (Table 2). The inoculated seedlings showed visually little fine root volume at the end of the experiment (Figure 3). Necrotic lesions were also observed in the roots, from the external to the internal parts (Figure 3). The plant roots were subjected to re-isolation and all stages of Koch's postulates were confirmed

FIGURE 3 | Pathogenicity of *Ilyonectria* isolates on *Pinus taeda* seedlings: (A) seedling without symptoms (control); (B) inoculated seedling with needle chlorosis; (C) dead inoculated seedling; (D) root necrosis in inoculated seedlings; (E) entry point wound to fungi; (F) healthy seedling root (control); (G, H) symptoms in the roots of seedlings inoculated with *I. leucospermi*; (I, J) symptoms in the roots of seedlings inoculated with *I. protearum* and (K, L) symptoms in the roots of seedlings inoculated with *I. robusta*; (M, N) symptoms in the roots of seedlings inoculated with *I. vredehoekensis*. (H, J, L, M) 10× magnification.

based on the morphological characteristics of the isolates obtained. No symptoms were observed in the 12 control plants.

4 | Discussion

This study identified four species of *Ilyonectria* associated with a decline in *P. taeda* trees in Brazil: *I. leucospermi, I. protearum, I. robusta* and *I. vredenhoekensis*. The pathogenicity of these species was confirmed on *P. taeda* seedlings. *Ilyonectria* species are known to be pathogenic to various woody plants, primarily causing root rot diseases (Erper et al. 2013; Dos Santos et al. 2014; Úrbez-Torres et al. 2014; Agustí-Brisach et al. 2016; Manici et al. 2018; Martínez-Diz et al. 2018; Mora-Sala et al. 2018; De Benedetti et al. 2024). This study provided strong evidence that these fungi affect the health of *P. taeda* trees.

The genus *Ilyonectria* belongs to the family Nectriaceae and consists of cosmopolitan fungi found in the soil and rhizosphere that can also be plant pathogenic (Chaverri et al. 2011; Summerell and Leslie 2011). The genus *Ilyonectria* was introduced by Chaverri et al. (2011) to separate and reclassify the asexual genus *Cylindrocarpon* and the sexual genus *Neonectria* (Booth 1959), where four new genera emerged: *Cylindrocarpon/Neonectria*, *Ilyonectria*,

TABLE 2 | Incidence of root and stem darkening, needle chlorosis and plant mortality in *Pinus taeda* seedlings inoculated with different *Ilyonectria* species after 180 days of incubation.

Isolate	Species	Incidence of darkening and chlorosis (%)	Incidence of mortality (%)
Control	—	0.0* c	0.0* b
VA1SPF6	*I. leucospermi*	91.7 a	8.3 b
VIIB3RF1	*I. leucospermi*	50.0 b	50.0 a
IIIM4RP1	*I. protearum*	66.7 a	33.3 b
VIIIA1RF1	*I. protearum*	33.3 b	66.7 a
IIIA3SP1	*I. robusta*	41.7 b	58.3 a
VA3RPF7	*I. robusta*	16.7 c	83.3 a
VIIB4RF3	*I. veredehoekensis*	33.3 b	66.7 a

*Averages followed by the same letter in the column do not differ statistically from each other by Scott Knott's 5% probability test.

Theleonectria and *Rugonectria* (Chaverri et al. 2011). For the reclassification, aspects such as the presence or absence of microconidia and chlamydospores, number of conidial septations and anatomy of the perithecia were considered (Chaverri et al. 2011). However, the genus *Ilyonectria* was distinguished from its sister genera based on its soil-borne nature and ability to produce microconidia and chlamydospores (Cabral, Rego, et al. 2012; Lombard et al. 2013). It was later shown by Lombard et al. (2014) that the genus *Ilyonectria* is paraphyletic, because of which a new genus (*Dactylonectria*) had to be designated to resolve this. At the same time, the genus *Cylindrodendrum* was shown to form a well-supported monophyletic sister clade to the *Ilyonectria* clade (Lombard et al. 2014).

Morphological characteristics are significant in describing fungal species (Taylor et al. 2000); however, they are not sufficient to distinguish between species within the genus *Ilyonectria* and related genera (*Cylindrocarpon*-like asexual morphs) (Cabral, Rego, et al. 2012; Lombard et al. 2014). In general, the morphological characteristics of the isolates analysed in this study align with the features described for each species identified. However, phylogenetic analysis is crucial for the identification and differentiation of species within a genus. Analysis of DNA sequences from the ITS region and partial sequences of HIS3 and TEF1 genes allowed for the identification of the seven isolates analysed in this study.

Morphological analysis of *Ilyonectria* isolates complemented the results of phylogenetic analysis. For all species, there was the formation of solitary conidiophores, microconidia, macroconidia with up to three septa and abundant chlamydospores, as described for this genus by Cabral, Groenewald, et al. (2012) and Lombard et al. (2013). Furthermore, sporodochia were formed in *I. protearum*, *I. vredenhoekensis* and *I. robusta*, and a visible hilum was present in the conidia of *I. leucospermi*, *I. protearum* and *I. vredenhoekensis*. Perithecia were not observed in any species in this study. Based on Chaverri et al. (2011), *Ilyonectria* perithecia are not commonly found in nature and are difficult to obtain in artificial media.

Fungi of the genus *Ilyonectria* are opportunistic necrotrophic pathogens that can often act as saprophytes attached to the surface of living roots. However, when stress occurs in the plant, triggering a reduction in resistance, these fungi exhibit phytopathogenic behaviour (Unestam et al. 1989; Menkis and Burokiene 2012).

Declines of nursery species, forest species, and woody agronomic plants by soil-transmitted asexual *Cylindrocarpon*-like morphs have been reported worldwide over the years (Agustí-Brisach and Armegol 2013; Haavik et al. 2015; Mora-Sala et al. 2018). In Spain, 16 species belonging to the genera *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria* and *Neonectria* were identified causing damage to the roots of 15 forest species in nurseries, in which *I. robusta* was identified causing damage to *Juglans regia* (Mora-Sala et al. 2018). In Uruguay, De Benedetti et al. (2024) reported *Cylindrocarpon*-like taxa causing decline in *Eucalyptus smithii* trees, among them a new species, *I. charruensis* sp. nov.

Species such as *I. radicicola, I. macrodidyma, I. alcacerensis, I. stremocensis, I. novozelandica, D. torresensis* and *I. robusta*, are widely distributed throughout the world and have been associated with the decline of grapevines (Cabral, Groenewald, et al. 2012). The disease, also named black foot of grapevine, occurs in seedlings in nurseries and plants already established in the field (Fourie and Hallleen 2001). In Brazil, there are only reports of three species of the genus *Ilyonectria* (*I. lirioodendri*, *I. macrodydima* and *I. robusta*) causing black foot disease of grapevine (Russi et al. 2010; Dos Santos et al. 2014). For this disease caused by *Ilyonectria* and *Dactylonectria* species, water stress and soil texture are important factors for pathogen infection (Probst et al. 2022). Clay soils are more likely to contract when water is not available, and thus damage and injure the roots, which serve as entry points for pathogens. Although lighter soils are less favourable to the occurrence of the disease, pathogens can survive in all types of soils (Probst et al. 2022). Therefore, growing vines in certain soil types does not eliminate this threat.

The species *I. robusta* is responsible for the reduction in root volume and death of temperate fruit trees, such as loquat (Agustí-Brisach et al. 2016), kiwi (Erper et al. 2013), and apple (Manici et al. 2018). This species has been reported to cause infection in *Codonopsis tangshen* (Zheng et al. 2022) in addition to infecting the roots of *Panax ginseng* in association with other species of *Ilyonectria* (Liu et al. 2019; Guan et al. 2020).

The species *I. leucospermi, I. protearum* and *I. vredehoekensis* have been identified as causing damage to cut flower plants of the Proteaceae family in South Africa (Lombard et al. 2013).

Likewise, the species *I. vredehoekensis* was also reported to cause damage to the roots of *Panax quinquefolius* (Zhang et al. 2019).

In this study, the species *I. leucospermi*, *I. protearum*, *I. robusta* and *I. vredenhoekensis* (not reported previously in Brazil) were found associated with adult *P. taeda* plants showing decline in Southern Brazil. Furthermore, they were shown to be pathogenic to *P. taeda* seedlings following artificial inoculation. To our knowledge, this is the first report of these species on *P. taeda* worldwide. However, to better understand the aetiology of plant decline caused by *Ilyonectria* spp., it is necessary to conduct complementary studies that examine both biotic and abiotic factors.

5 | Conclusions

The species *Ilyonectria leucospermi*, *I. protearum*, *I. robusta* and *I. vredenhoekensis* are pathogenic to *P. taeda* and are involved in the aetiology of pine tree decline in Southern Brazil.

Author Contributions

Ana Carolina Lyra Brumat: conceptualisation, methodology, formal analysis, investigation, visualisation, writing – original draft. **Celso Garcia Auer:** conceptualisation, methodology, visualisation, writing – review and editing. **Dauri José Tessmann:** methodology, formal analysis, visualisation, writing – review and editing. **Caroline de Bastos Bührer:** methodology, investigation, writing – review and editing. **Henrique da Silva Silveira Duarte:** conceptualisation, methodology, visualisation, writing – review and editing. **Álvaro Figueredo dos Santos:** conceptualisation, methodology, visualisation, writing – review and editing. All authors contributed equally to the design and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.