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Thesis

IDENTIFICATION, DIAGNOSIS, AND STRATEGIES FOR MANAGEMENT OF FUNGAL DISEASES CAUSING LEAF SPOTS ON *Lolium multiflorum* IN SOUTHERN BRAZIL

Alfonso Daniel Victoria Arellano

Pelotas, 2020

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Identification, diagnosis, and strategies for management of fungal diseases causing leaf spots on *Lolium multiflorum* in southern Brazil

Thesis submitted to the Crop Protection Graduate Program of the Faculty of Agronomy Eliseu Maciel of the Federal University of Pelotas, as a partial requirement for obtaining the title of Doctor of Science (Area: Plant Pathology).

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Dedicated to the four women who defined my life, to whom I owe what I am and whom I love and admire with all my being

My grandmother Micaela (in memory)

My mother Estela

My aunt Nestor

My girlfriend Karina

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"Que les coses siguin d'una manera, no vol dir que no es puguin canviar"

Merlí Bergeron

Resumo

Victoria Arellano, AIfonso Daniel. **Identificação, diagnóstico e estratégias de manejo de patógenos fúngicos causadores de manchas foliares em *Lolium multiflorum* no sul do Brasil. 2020, 125f. Tese** (Doutorado). – Programa de Pós-graduação em Fitossanidade, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, 2020.

O diagnóstico fitossanitário de fungos que causam manchas nas folhas de *Lolium multiflorum* se tornou relevante nos últimos anos porque afeta indiretamente a criação de gado, que é uma das principais atividades econômicas do sul do Brasil. Esta pastagem contribui bastante para as necessidades alimentares deste setor nos períodos de inverno devido à disponibilidade e qualidade nutricional. As doenças fúngicas que causam manchas nas folhas foram identificadas como o principal problema que afeta a produção nessa forragem. Atualmente, a identidade e a importância desses patógenos são desconhecidas e não existem medidas recomendadas para seu manejo. Portanto, este estudo teve como objetivo identificar os principais fungos associados a essas doenças e avaliar estratégias de manejo para reduzir a intensidade das doenças, por meio da indução da resistência e redução da fonte de inóculo. Para esse fim, a identidade taxonômica dos patógenos foi revelada a partir de uma abordagem polifásica, e sua patogenicidade foi testada pelos Postulados de Koch. Posteriormente, foram testadas estratégias baseadas na incorporação de silício no solo (Si), inoculação de sementes com *Trichoderma atroviride* (T) ou ambas (TSi) contra *Pyricularia oryzae* (em casa de vegetação). Finalmente, essas estratégias foram testadas em campo, juntamente com o manejo da frequência de corte (C), no controle das doenças com ocorrência natural de inóculo. Foram identificadas sete espécies de fungos associados a manchas foliares, das quais *P. oryzae* e *Bipolaris sorokiniana* foram novos relatos no Brasil, *Pyrenophora nobleae*, *Colletotrichum plurivorum* e *Exserohilum rostratum* foram determinadas como novas associações no hospedeiro, enquanto *Curvularia* sp. nov. e *Cercospora* sp. nov. eles são apresentados como duas novas contribuições para a ciência. Quanto ao manejo, verificou-se que a aplicação de *T. atroviride* nas sementes e a aplicação de silício no solo resultaram em proteção contra *P. oryzae*. Também foram detectadas a brusone (*P. oryzae*), a mancha marrom (*B. sorokiniana*) e a mancha ocular do papagaio (*Cercospora* sp. nov.) como as principais doenças associadas a *L. multiflorum*. As estratégias T, Si, TSi e C reduziram a severidade da mancha marrom e da brusone em 30 e 65%, respectivamente. Esses resultados formam uma base para o diagnóstico de patógenos que causam manchas nas folhas de *L. multiflorum*, além de fornecer estratégias viáveis de manejo, reduzindo assim os danos de maneira sustentável.

Palavras chave: Grama forrageira, abordagem polifásica, manejo integrado de doenças, doenças de azevém, indução de resistência.

Abstract

Victoria Arellano, AIfonso Daniel. **Identification, diagnosis and management strategies of fungal pathogens that cause leaf spots in *Lolium multiflorum* in southern Brazil. 2020, 125s.** Thesis (Doctorate) –Crop Protection Graduation Program, Faculty of Agronomy Eliseu Maciel, Federal University of Pelotas, Pelotas, 2020.

The phytosanitary diagnosis of fungi that cause leaf spots in *Lolium multiflorum* has become very relevant in recent years because it indirectly affects cattle feeding, which is one of the main economic activities in southern Brazil. This pasture contributes highly to the food needs in this sector in winter periods due to the availability and nutritional quality. Fungal diseases that cause leaf spots have been identified as the main problem affecting production in this forage. Currently, the identity and importance of these pathogens are unknown and there are no recommended measures for their management. Therefore, this study aimed to identify the main fungi associated with these diseases and to evaluate management strategies to reduce disease intensity by inducing resistance and reducing the inoculum source. To this end, the taxonomic identity of pathogens was revealed from a polyphasic approach and their pathogenicity was tested through Koch's Postulates. Subsequently, strategies based on the incorporation of silicon in the soil (Si), seed inoculation with *Trichoderma atroviride* (T), or both (TSi) against *Pyricularia oryzae* (in a greenhouse) were tested. Finally, these strategies, along to the management of the cutoff frequency (C), were tested in the field against naturally occurring diseases. Seven species of fungi associated with leaf spots were identified, of which *P. oryzae* and *Bipolaris sorokiniana* were new reports in Brazil, *Pyrenophora nobleae*, *Colletotrichum plurivorum*, and *Exserohilum rostratum* were determined as new associations in the host, while *Curvularia* sp. nov. and *Cercospora* sp. nov. they are presented as two new contributions to science. Concerning management, it was found that the application of *T. atroviride* in the seeds and the application of silicon in the soil resulted in protection against *P. oryzae*. Also, the gray leaf spot (*P. oryzae*), brown leaf spot (*B. sorokiniana*) and parrot eye leaf spot (*Cercospora* sp. nov.) were detected as the main diseases associated with *L. multiflorum*. The T, Si, TSi, and C strategies reduced the severity of brown leaf spot and gray leaf spot in 30 and 65%, respectively. These results form a basis for the diagnosis of pathogens that cause leaf spots in *L. multiflorum*, as well as providing viable strategies for management, thus reducing damage sustainably.

Key words:

Forage grass, polyphasic approach, integrated disease management, ryegrass diseases, resistance induction.

SUMMARY

INTRODUCTION

The cattle ranching has become one of the main activity of the Brazilian economy (Calixto and Hespanhol 2014), with a total effective of 173 million heads and 30 billion liters of milk, of which 6.6% of cattle and 13% of milk were produced in the state of Rio Grande do Sul in 2017(IBGE 2020). In this region, the Italian ryegrass (*Lolium multiflorum* L.) has become one of the most important plants in the livestock production sector due to nutritional advantages, easy establishment and availability of food during winter (Aguinaga et al. 2006; Lopes et al. 2008; Hoffmann et al. 2014).

In recent years, diseases that attack *L. multiflorum* have become a priority in breeding programs, since forage yield is directly related to the presence of diseases that end up reducing dry matter and seed production (Donizeti and Nunes 2009). In this host, 153 species of pathogenic fungi have been registered in the world, and only seven of them have been reported in Brazil (Farr and Rossman 2020). However, some studies have revealed the presence of more than 20 species of fungi associated to seeds of *L. multiflorum* in this country (Silva et al. 2014); therefore, there is a lack of information about the diseases leaf spots that occur in this plant. The proper identification and classification of plant disease-causing fungi are critical to the establishment of effective quarantine regulations, fungicide application protocols, and breeding programs that target disease resistance (Liu et al. 2019). Furthermore, there are no fungicides legally approved by the Brazilian Ministry of Agriculture against any pathogens in this forage grass (AGROFIT 2020), and pesticides are currently one of the biggest public health problems in Brazil due to the size of the population exposed in agricultural areas (Rigotto et al. 2014). In this scenario, it is necessary to create alternatives to improve forage yields with efficient and ecological strategies.

There are several alternatives for integrated disease management (IDM) that aim to reduce the amount of disease while maintaining a stable relationship with the environment. One of the most studied is the use of biological control agents, in which species of *Trichoderma* are among the most widely used (Ali Nusaibah and Musa 2019). Species in this genus acts by activating single or multiple control mechanisms, including mycoparasitism, enzyme production, competition (nutrients/space), and induced resistance in plants that, directly or indirectly, interfere with the target pathogen (Ali Nusaibah and Musa 2019).

Furthermore, silicon is another alternative that has been reported to improve the defense of plants against pathogens caused by fungi, bacteria, and viruses (Luyckx et al. 2017), through physical-mechanical resistance, induction of biochemical and molecular responses (Debona et al. 2017; Wang et al. 2017). However, there are also cultural practices within the IDM scheme that aim to reduce the disease through management of the inoculum source. In this sense, planning of pasture supply, which guarantees forage during periods of scarcity and allows better management of food quality (Santos et al. 2009) could be a timely strategy to reduce the amount of disease. Therefore, this study aimed: 1) to carry out the identification of pathogens associated with diseases that cause leaf spots in *Lolium multiflorum*, 2) to determine the importance of these pathogens infection in natural conditions, and 3) to evaluate strategies aiming to reduce inoculum source (pasture cutting) and induce host resistance (application of silicon and or *Trichoderma spp*.) to reduce the intensity of leaf spots.

The research was divided into three studies: In the first one, it contemplated the identification of fungi (under the polyphasic approach) associated with leaf spot diseases in *L. multiflorum.* Isolation of pathogens was carried out from leaf tissue samples with symptoms of spots in grazing areas of Rio Grande do Sul and were tested using Koch's postulates.

The second study aimed at the isolation and evaluation of rhizospheric strains of *Trichoderma* spp. to find a possible resistance inducer, and with the ability to directly or indirectly inhibit *Pyricularia oryzae*. Additionally, the response of *Trichoderma atroviride* and the incorporation of silicon in the soil were evaluated as alternatives to reduce the severity of the gray leaf spot caused by *P. oryzae* in greenhouse conditions.

The third part of the work considered the same treatments evaluated under greenhouse conditions along with the implementation of different cutoff frequencies of the pasture (aiming at reducing the source of the inoculum) to evaluate these management alternatives to reduce the incidence and severity of diseases of leaf spots in *L. multiflorum* field conditions.

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Fungi causing leaf spots diseases on *Lolium multiflorum* in Brazil

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Abstract

Leaf spots diseases caused by fungi presents a serious threat to forage crops, due to the reduced photosynthetic area, dramatically affecting harvest and total yields. Aiming to expand the knowledge of fungi causing leaf spot diseases on ryegrass (*Lolium multiflorum*) from southern Brazil, a comprehensive survey was performed, which resulted in seven species of plant pathogenic fungi. All species have been identified using a polyphasic approach, combining morphological, ecological and molecular sequence data from, when necessary, up to seven different genomic loci (actin, calmodulin, glyceraldehyde 3-phosphate dehydrogenase, histone, ITS, LSU and partial translation elongation factor 1-α). Representative strains of each genus were subjected to pathogenicity tests. Among all species studied, two represents new-host association (*Exserohilum rostratum* and *Colletotrichum plurivorum*), three are new reports to Brazil (*Bipolaris sorokiniana*, *Pyricularia oryzae*, and *Pyrenophora nobleae*), and two are new to science, namely *Curvularia* sp. nov., and *Cercospora* sp. nov, which were described and illustrated. The results presented here forms the basis for identifying at the field, which in the future, could help breeders to design strategies for the control and integrated management in crop-livestock production systems.

Key words: forage crop, multilocus sequencing, mycobiota, ryegrass disease

Introduction

In 2017, the Brazilian livestock sector represented 8.7% of its gross domestic product, and consisted of 173 million head and 160 million ha of pasture, of which 11.5 million head of cattle and 9 million hectares were concentrated in Rio Grande do Sul (IBGE 2020). One of the most widely used forage crops in this region is the italian ryegrass (*Lolium multiflorum* Lam. Poaceae), especially in the winter due to its nutritional characteristics and adaption to crop-livestock production systems (Tonetto et al. 2011; Silva et al. 2014).

The genus *Lolium* comprises about eight recognized species (mostly annual herbs), and five hybrids, however, the two most important ryegrass species are Italian ryegrass (*L. multiflorum*) and perennial ryegrass (*L. perenne*), which widely grow as forage and cover crops as well as turf grasses in Europe, New Zealand, Australia, North and South America (Polok 2007).

L. multiflorum is an annual plant of up to 1.5 meters, with erect, cylindrical culms (composed of nodes and internodes), thin leaves, short and membranous ligule, present or absent auricles, lanceolate to oblong lemma, smooth and membranous palea, distal inflorescence and compressed lateral spikelet's attached to the stem (Polok 2007).

In temperate environments, the breeding of ryegrass has been carried out aiming to obtain cultivars with cold tolerance, the increase in the production of forage and its nutritional components and the increase in the production of seeds (Flores et al. 2008), however, for the last decade, fungi diseases of *L. multiflorum* have become a priority in breeding programs, since forage yield can be significantly harmed by their attack, which end up reducing dry matter and seeds production (Donizeti and Nunes 2009). On the concernment of fungi pathogens related to *L. multiflorum*, 153 species have been recorded worldwide, but only seven are known from Brazil (Farr and Rossman 2020). Although some studies have revealed the presence of more than 20 species of fungi related to *L. multiflorum* seeds (Silva et al. 2014), and few species that might poses as potentially dangerous to the crop (Lucca Filho et al. 1999), little is known about the diversity of pathogens that are occurring in ryegrass fields from Brazil, especially in Southern region, where it is largely planted. Leaf spots diseases in *L. multiflorum* have been devastating in other countries, such as the gray leaf spot, caused by *Pyricularia* sp., in Japan (Miura et al. 2005). The disease can affects the seedlings, leading the plant to death in a matter of days (Takahashi et al. 2014). In Brazil, it has been associated with *Pyricularia griseae* (Donizeti et al. 2017), based solely on morphological data, and therefore, not precise for a correct identification (Manamgoda et al. 2014; Tan et al. 2014). Proper identification and classification of plant-pathogens are critical to the establishment of effective quarantine regulations, fungicide application protocols, and breeding programs that target disease resistance (Liu et al. 2019). The use of a polyphasic approach, such as the consolidated species concept (Quaedvlieg et al. 2014) integrates ecology, morphology, cultural characteristics and multilocus DNA phylogenetic data, in order to appropriately verify species boundaries, providing a better understanding of diversity (Das et al. 2014).

The present survey details the identification of fungi causing leaf spots in *L. multiflorum*, at two different locations, in southern Brazil in the light of the consolidated species concept as well as to investigate the aggressiveness and pathogenicity of each species found in order to evaluate its destructive potential.

Materials and methods

Specimens and isolates

Leaf tissue samples from the cultivar BRS Ponteio were collected in two different areas located within the Pampa biome, namely the Agricultural Center of Palma / Federal University of Pelotas, in the municipality of Capão do Leão, and in the facilities of the Brazilian Company for Agricultural Research (EMBRAPA), in the municipality of Bagé. Plant bearing leaf spots were randomly collected in the studies areas in plastic bags and then stored at 6 °C, for no more than 48 h until analysis. Whenever possible, single-conidia colonies were established on potato dextrose agar (PDA) from fungal structures, hyphae and/or conidia scraped from the surface of the lesions. In the absence of direct isolation, a portion of the infected tissue was placed in a plate containing agar–water (AA), and after growth, isolation was performed thought transfer of hypha to a new plate with PDA, malt extract agar (MEA) or oatmeal agar (OA) (Crous et al. 2019). Fungal structures were mounted in clear lactophenol or cotton blue. Observations, measurements and images were made with a light microscope (LEICA, Mod. DM1000) coupled to camera (LEICA DFC295) and Leica Application Suite Core (LAS V3.7). The intervals were generated from 30 observations, extreme dimensions are given in parentheses. Colony descriptions were based on observations of colonies formed on PDA, MEA and OA in dark at 25 °C and in a 12 hours dark/light regime, for seven days. Taxonomic novelties were deposited in MycoBank (www.MycoBank.org; Crous et al. 2004).

Pathogenicity tests

Pathogenicity of representative strains were carried out individually for each strain. Strains were grown in PDA or OA for 7 d at 26 °C, with 12-hour photoperiod. Subsequently, healthy plants of 60-day old (tillering stage) of cultivar BRS Ponteio were inoculated with a suspension of 10^5 conidia mL^{-1}, and transferred to a bench in a greenhouse at 22 to 29 °C, and relative humidity of 90%. Plant were checked for symptoms each 12 hours. Plants sprayed with water served as controls.

DNA isolation, amplification, and sequencing

Isolates were grown on PDA plates for 10 d at 26 °C. Genomic DNA was extracted from mycelium using the DNA isolation kit (Norgen, Ontario, Canada), following the manufacturer´s instructions. Seven partial regions were initially targeted for PCR amplification an sequencing, namely actin (*act*), calmodulin (*cmdA*), histone (*his3*), internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS) of the nrDNA operon, glyceraldehyde 3-phosphate dehydrogenase *(gapdh*), 28S nrRNA gene (LSU) and, the partial translation elongation factor 1-α (*tef1*). PCR amplifications were performed in an automated thermal cycler (MJ Research PTC–200), using a PCR master mix (Thermo Fisher Scientific Massachusetts, USA). The reaction was made at final volume of 25 µL, containing 12.5 µL of PCR master mix, 1 uL (10 pmol) of each primer (forward and reverse),1 uL of DNA (30 ng/µL) and 9.5 uL of sterile distilled water. Primers and PCR programs used for the amplification are described in Table 1. PCR products were visualized on electrophoresis gel (1% agarose gel) stained with Diamond Nucleic Acid Dye (Promega, Wisconsin, USA). Sizes of the amplified DNA were obtained using the DNA ladder marker (Invitrogen). PCR products were purified with a purification Kit (Mebep Bioscience, Shenzhen, China) and stored at -20 °C, until sequencing. PCR fragments were sequenced in both directions using the same primers used for amplifications and the BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems,

USA), following the manufacture´s recommendations. The products were analyzed on an ABI Prism 3730 XL DNA Sequencer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences in the MEGA (Molecular Evolutionary Genetics Analyses) v. 7.0 (Kumar et al. 2016) and were manually edited whenever necessary. Megablast searches of nucleotide database were compared with each generated consensus sequences. Each locus was analyzed separately to determine preliminary identifications of the isolates.

Phylogenetic analysis

Consensus sequences were generated and imported into MEGA v. 7.0 for initial alignment and the construction of sequence datasets. For initial alignment and the construction of sequence datasets. Initially, sequences obtained from the previous datasets were used in each analyze. Manamgoda et al. (2014, 2015) for *Bipolaris*; Damm et al. (2019) for *Colletotrichum*; Hernández–Restrepo et al. (2018) for *Exserohilum*; Marin–Felix et al. (2019) for *Pyrenophora*, Bakhshi et al. (2018) for *Cercospora*, and Klaubauf et al. (2014) for *Pyricularia* (as listed in Tables 2-8). To each of these analysis was added the novel sequences generated during this study and then aligned using MAFFT v. 7 (Katoh et al. 2019). After a preliminary analysis, the datasets were trimmed down to Brazilian isolates and the direct neighbours.

Appropriate gene models were selected using MrModeltest v. 2.3 (Nylander 2004) and applied to each gene partition. Based on the results of MrModeltest, a Bayesian phylogenetic analysis was performed with MrBayes v. 3.2.1 (Ronquist et al. 2012) applying different substitution models for each locus as listed in Table 9. Bayesian inferences were performed using MrBayes on XSEDE v. 3.2.6 on CIPRES (www.philo.org). Six simultaneous Markov chains were run for 10,000,000 generations and trees were sampled every 1,000th generation, until convergence (stopval = 0.01) was reached. Burn-in was set at 25 % and the remaining trees were used to calculate posterior probabilities (PP). A heating parameter ('temp') of 0.15 was used for all genera analyzed (Guatimosim et al. 2016). The trees were reviewed and adjusted in Geneious 9.0.5 and customized in Adobe Illustrator CS5.1. Novel sequences derived in this study were lodged at GenBank and the alignments and phylogenetic trees in TreeBASE (www.treebase.org/treebase/index.html).

Results

Phylogenetic analyses

Seven different datasets were constructed and consisted of 1,842 characters for *Bipolaris* (477 for ITS, 895 for *tef1*, 470 for *gapdh*), 1,185 characters for *Cercospora* (457 for ITS, 169 for *act*, 235 for *cmdA*, 324 for *his3*), 1,562 for *Curvularia* (499 for ITS, 508 for *gapdh*, 555 for *tef1*), 1,211 characters for *Colletotrichum* (542 for ITS, 419 for *his3*, 250 for *act*), 1,090 characters for *Exerohilum* (491 for ITS, 599 for *gapdh*), 2,197 characters for *Pyrenophora* (668 for ITS, 677 for *gapdh*, 852 for LSU) and 1,288 characters for *Pyricularia* (473 for ITS, 307 for *act*, 508 for *cmdA*).

The number of taxa (including the outgroup), unique site patterns for each locus, total unique site patterns, total trees generated and sampled (considering, a burn-in fraction of 0.25), for each phylogenetic study, are presented in Table 10. In each analysis the posterior Bayesian probabilities (PP) are presented to the left of each node (Figures 2, 4, 6, 8, 10, 12, 14).

Taxonomy

One hundred and twenty samples of *L. multiflorum* leaf spots were collected on the surveyed areas. From those, 29 strains were isolated and later identified in seven fungal species, including *Bipolaris sorokiniana*, *Colletotrichum plurivorum*, *Exerohilum rostratum*, *Pyrenophora nobleae*, and *Pyricularia oryzae*. *Cercospora* sp. nov. and *Curvularia* sp. nov. are introduced as new to science.

Bipolaris sorokiniana (Sacc.) Shoemaker, Can. J. Bot. 37: 884. 1959 – Fig. 1

Description in planta – Leaf spots rounded, oval or effusing parallel to the margin of the leaf, forming large patches, coalescing and becoming necrotic. For a complete description and illustration, see Shoemaker (1959).

Specimens examined. Brazil, Rio Grande do Sul, Capão do Leão, Agricultural Center of Palma, field, on leaves of *Lolium multiflorum*, 5 Nov. 2016, A.D. Victoria-Arellano (LIPP, cultures LIPP BI4, LIPP BI1, LIPP BI2, LIPP BI3, LIPP BI4).

Notes – *Bipolaris sorokiniana* is known causing disease on *L. multiflorum* from Australia (Sivanesan 1987) and Brazil (Silva et al. 2014). The Brazilian report however, drives from a simple experiment, in which the authors isolated *B. sorokiniana* from seeds of *L. multiflorum*. Additionally, the authors identified the fungal species based solely on morphology, which is known to be not sufficient to delimitate species boundaries in *Bipolaris* (Manamgoda et al. 2014). Phylogenetically, the isolate studied here, clusters within the *B. sorokiniana* clade, alongside with others strains from different countries of the world (Fig. 2). Therefore, based on morphology and molecular data, to our knowledge, this is the first report of *B. sorokiniana* causing leaf spots on *L. multiflorum* from Brazil.

***Cercospora* sp. nov.** Victoria-Arellano, Guatimosim & Dallagnol, sp. nov. – Mycobank MB (code will be assigned) – Fig. 3

Description in planta – *Leaf spots* rounded, elliptical or irregular, scattered on the width or on the margins. *Internal hyphae* hyaline. *Caespituli* amphigenous, emerging from epidermal cells and through the stomata. Stroma substomatal, amphigenous, pale brown to dark brown at the base, up to 30 µm diam. *Conidiophores* forming fascicles (5–10 stalk per fascicle), straight to slightly curved, flexuous, (54) 60–313 (316) × (1) 2–5 (6) µm, unbranched, 4–10–septated, pale brown to brown, smooth, slightly sinuous and thickened at the base, truncated apex, scars conspicuous, thickened and darkened. *Conidia* solitary, acicular, hyaline to slightly grayish, (171)133–351(322) × (3)2.5–5(4.8) µm, base truncated, 2.5–4.5 µm at the base, 8–22–septated, hila slightly thickened and darkened. *Sexual morph* not observed. Culture characteristics – Colonies on PDA and OA, slow growing, 30 mm diam after 7 d, circular, with white cottony mycelium, soft gray margins, flat; culture sporulating. Under photoperiod wavy margin with a slightly white border. On MEA under photoperiod, slightly pigmenting the media in violet.

Specimens examined: Brazil, Rio Grande do Sul, Capão do Leão, Agricultural Center of Palma, field, on leaves of *Lolium multiflorum*, 5 Nov. 2016, A.D. Victoria-Arellano (LIPP, culture ex-type LIPP C2A, isotypes LIPP C1C. LIPP C4B, LIPP C2A, LIPP C1B, LIPP C3C).

Notes – Despite the large number of *Cercospora* species reported from monocots and Poaceae (Braun et al. 2014, 2015a), there is not a single species recorded on *Lolium*. Phylogenetically, *Cercospora* sp. nov. grouped basal to the tree, having *Cercospora* sp. G (sensu Groenewald et al. (2013) as sister clade (Fig. 4). The *Cercospora* sp. G was recently recognized as a complex, since in the 8-gene phylogenetic analysis (ITS, *tef1, act, cmdA, his3, tub2, rpb2* and *gapdh*), it clustered into two distinct phylogenetic clades (Bakhshi et al. (2018). Nevertheless, based on the combined 4-gene analysis (ITS, *act, cmdA* and *his3*), *Cercospora* sp. nov. can be recognized as a separate species.

Colletotrichum plurivorum Damm, Alizadeh & Toy. Sato, Stud. Mycol. 92: 31. 2019 – Fig. 5

Description in planta – *Leaf spots* starting as small necrotic irregular lesions, when mature, developing a small, whitish space in the center of the lesion, and sporulating. At later stages, leaf becomes chlorotic at plant anthesis stage, disease can cause necrotic lesions accompanied by glume spore mass. For a complete description and illustration, see Damm et al. (2019).

Specimens examined: Brazil, Rio Grande do Sul, Bagé, Embrapa Pecuária Sul, field, on leaves of *Lolium multiflorum*, 27 Oct. 2017 A.D. Victoria-Arellano and L.J. Dallagnol (LIPP, cultures LIPP COLL1B, LIPP COLLL1C, LIPP COLL1B, LIPP COLL2B, LIPP COLL2C, LIPP COLL3B).

Notes – *Colletotrichum plurivorum* can cause disease in a wide range of hosts, including Anacardiaceae, Araceae, Caricaceae, Fabaceae, Malvaceae, Musaceae, Orchidaceae, Passifloraceae, Rosaceae, Rubiaceae, Solanaceae, and Theaceae, being known from Benin, Brazil, China, India, Iran, Japan, Malaysia, Mexico, Myanmar, Taiwan, Thailand, and Vietnam (Damm et al. 2019). Three other species of *Colletotrichum* are known causing disease on *Lolium*, namely *C. cereale* on *L. perenne*, from South Africa, *C. dematium* on *L. loliaceum*, from Australia, and *C. graminicola* on *L. multiflorum*, *L. perenne*, *L. rigidum*, and *L. temulentum*, from a wide range of countries (Farr & Rosmann 2020). Phyllogenetically, *C. plurivorum* belongs to the *C. orchidearum* species complex (Damm et al. 2019), having *C. cliviicola* as sister clade (Fig. 6), while *C. graminicola*, known on *L. multiflorum* from USA, belongs to a different species complex (Tao et al. 2013). This is the first report of *C. pluvirovum* causing leaf spots on *L. multiflorum* worldwide, including Brazil.

***Curvularia* sp. nov.** Victoria-Arellano, Guatimosim & Dallagnol, sp. nov. – Mycobank MB (code will be assigned) – Fig. 7

Description in planta – *Leaf spots* initially irregular at the margins or within the atrium, starting at the base or somewhat upper on the leaves, sometimes limited by the central vein. On PDA – *Conidiophores* erect, straight to flexuous, geniculated towards the apex, (60) 77–223 (235) × (4.5)6–11(13) µm, brown at the base, paler towards the apex, septated, smoth. *Conidiogenous cells* integrated, terminal or intercalary, sympodial, pale brown to brown, smooth, scars thockned and darkened. *Conidia* obovoid to ellipsoide, straight to curved, (26–) 29–45 (–48) × (11–)13–21 (–23) µm, terminal cells brown to light brown at the apex, third cell (anterior to upper apex) often more swollen, 3-distoseptate, hila protuberant, thickened and darkened, 1-3 µm wide. *Sexual morph* not observed.

Cultural characteristics – Colonies on PDA, under photoperiod, fast growing, 71 cm diam after 7 d, circular, white cottony aerial mycelium, centraly pale brown, alightly white at the margins, flat; sporulating abundantly. On MEA, under photoperiod, fast growing, 62 cm

diam after 7 d, margins entire, convex elevation, aerial mycelium centrally gray, pale brown at the margins; sporulating abundantly. On OA, under photoperiod, culture sterile.

Specimens examined: Brazil, Rio Grande do Sul, Bagé, Embrapa Pecuária Sul, field, on leaves of *Lolium multiflorum*, 27 Oct. 2017, A.D. Victoria-Arellano and L.J. Dallagnol (LIPP, culture ex-type LIPP CU1, isotype LIPP CU2).

Notes – Three species of *Curvularia* are known causing diseases on *L. multiflorum*, namely *C. intermedia*, *C. lunata*, and *C. trifolli* (Farr & Ropssman 2020). Of these, only *C. intermedia* has molecular data available (Li et al. 2019) and besides it is only ITS, it is quite different from *Curvularia* sp. nov. (Fig. 8). *Curvularia lunata* differs from the Brazilian isolate by having longer conidiophores, and smaller conidia (Manamgoda et al. 2012). Additionally, it is only known attacking *L. multiflorum* from USA (Sprague 1959). In the other hand, *C. trifolli* differs from *Curvularia* sp. nov. by having mostly geniculate conidiophores and smaller (Crous et al. 2011; Khadka 2016; Coelho et al. 2020). Moreover *C. trifolli* is only known causing leaf spots on *L. multiflorum* from New Zealand and Portugal (Sivanesan 1987, Pennycook 1989).

Exserohilum rostratum (Drechsler) K.J. Leonard & Suggs, Mycologia 66: 290. 1974 – Fig. 9

Description in planta – Lesions usually at the margins of the leaf, appearing as small semi-elliptic spots, becoming irregular when mature, and rarely limited by a chlorotic halo. Sporulating adaxially. For a full description and illustrations, see Hernández-Restrepo et al. (2018).

Specimens examined: Brazil, Rio Grande do Sul, Bagé, Embrapa Pecuária Sul, field, on leaves of *Lolium multiflorum*, 5 Nov. 2016, A.D. Victoria-Arellano & L.J. Dallagnol (LIPP, cultures, LIPP EX1, LIPP EX2).

Notes – *Exserohilum rostratum* is a cosmopolitan species recorded on numerous hosts, from several countries, especially *Poaceae* and other monocots, but also as human pathogens (Hernández-Restrepo et al. 2018). On the host genus *Lolium*, however, there are no records of *E. rostratum* causing diseases worldwide (Farr & Rossman 2020). Based on the Bayesian analysis of the combined ITS-*gapdh* (Fig. 10), the Brazilian isolate on *Lolium*, clusters within the *E. rostratum* clade. The present study did not reach at the lineage level as proposed by Hernández-Restrepo et al. (2018), once it was out of the scope. To the best of our knowledge, this is the first report of *E. rostratum* on *L. multiflorum* worldwide, including Brazil.

Pyrenophora nobleae (McKenzie & D. Matthews) Rossman & K.D. Hyde, IMA Fungus 6: 518. 2015 – Fig. 11

Description in planta – Lesions at the base of the leaves, or slightly upper, apperaring as rounded necrotic spots, 2 mm wide, becoming elongated to irregular. For a complete description and illustration, see Rossman et al. (2015).

Specimens examined: Brazil, Rio Grande do Sul, Bagé, Embrapa Pecuária Sul, field, on leaves of *Lolium multiflorum*, 17 Oct. 2016, A.D. Victoria-Arellano and L.J. Dallagnol (LIPP, cultures, LIPP DC1, LIPP DC2).

Notes – Eight species of *Pyrenophora* have been recorded on *L. multiflorum* namely, *P. avenae*, *P. biseptata*, *P. dematioidea*, *P. dictyoides*, *P. lolii*, *P. nobleae*, *P. poae*, and *P. teres*, but none from Brazil (Farr & Rossman 2020). *Pyrenophora nobleae* have been recorded on *L. multiflorum* from Australia, New Zealand, UK and USA (Morrison 1982; Sivanesan 1987; Pennycook 1989). Based on the multi-gene phylogenetic inference (Fig. 12), the Brazilian isolate clustered within the *P. nobleae* clade, together with strains from Germany (CBS 259.80 and CBS 966.87). Despite these strains are not ex-type, they are considered as representative for the species (Marin-Felix et al. 2018). This is the first report of *P. nobleae* causing leaf spot disease on *L. multiflorum* from Brazil.

Pyricularia oryzae Cavara , Fung. Long. Exsicc. 1: no. 49. 1891. Fig. 13.

Description in planta – Lesions starting as necrotic spots that eventually develop into large ellipsoidal, semicircular, irregular, or typically diamond–shaped spots. Symptoms on the spikes appeared on the rachilla, usually with a dark brown to black lesion accompanied by greyish sporulation. In response, the spike turns white from where the lesion originates, causing seed sterility. For a full description and illustration, see Klaubauf et al. (2014).

Specimens examined: Specimens examined: Brazil, Rio Grande do Sul, Bagé, Embrapa Pecuária Sul, field, on leaves of *Lolium multiflorum*, 5 Nov. 2016, A.D. Victoria-Arellano (LIPP, cultures LIPP PY2, LIPP PY1, LIPP PY2, LIPP PY3, LIPP PY5). *Ibidem*, on leaves of *Lolium multiflorum*, 11 Nov. 2016, A.D. Victoria-Arellano and L.J. Dallagnol (LIPP, cultures LIPP PYB1, LIPP PYB2, LIPP PYB4, LIPP PYB5).

Notes – *Pyricularia oryzae* have been reported causing disease on *L. multiflorum* from China, USA and Uruguay (Tosa et al. 2007; Xue et al. 2017; Yasuhara-Bell et al. 2018). In Brazil, it has been associated only with *L. perenne*, *Oryza sativa* and *Triticum aestivum* (Murata et al. 2014). In the Bayesian analysis of the combined 3-gene sequence alignment (ITS, *act* and *cmdA*), the Brazilian isolate clusters within the well-established clade of *P. oryzae* (Fig. 14). This is the first record of *P. oryzae* causing leaf spots on *L. multiflorum* from Brazil.

Discussion

The present survey aimed at identify plant pathogenic fungi causing leaf spots on *L. multiflorum* in Southern Brazil. In the light of the consolidated species concept, seven species were recognized as pathogens of *L. multiflorum* (cultivar BRS Ponteio), from which two are proposed as new to science.

Bipolaris sorokiniana has a large number of hosts within Poaceae, including not only important crops, such as *Avena sativa*, *Hordeum vulgare*, *Oryza sativa*, *Secale cereale*, *Triticum eastivum*, and *Zea mays*, but also different commonly used pastures, such as *Brachiaria plantaginea*, *Echinochloa crus-galli*, and *Festuca arundinacea* (Farr and Rossman 2020). That being said, the crop rotation with *L. multiflorum* should be used with caution, once cross infection of *B. sorokiniana*, isolated from different hosts, are no unusual (Ramos et al. 2003).

The genus *Cercospora* have been thoroughly studied in the past two decades by both morphological characters (Crous and Braun 2003; Braun et al. 2014, 2015a, b, 2016; Albu et al. 2016) and molecular biology (Schoch et al. 2009; Groenewald et al. 2013; Bakhshi et al. 2015; Soares et al. 2015; Albu et al. 2016; Guatimosim et al. 2016; Guillin et al. 2017). The

Consolidated Species Concept adopted here (Quaedvlieg et al. 2014), is proved as an effective method for defining species boundaries in *Cercospora* (Groenewald et al. 2013). Based on morphology, *Cercospora* sp. nov. does not coincide with any previously described species. Additionally, based on the phylogenetic analysis (Fig. 4) *Cercospora* sp. G (sensu Groenewald et al. 2013) clusters as a sister clade, but differs from *Cercospora* sp. nov. with high supporting posterior probability (pp=1).

Colletotrichum plurivorum is known to cause disease on a large number of hosts, in fact more than 20 hosts have been reported (Douanla-Meli et al. 2018; Damm et al. 2019; Liu et al. 2019). Future evidence is needed to know if the strain isolated on *L. multiflorum* is pathogenic to *Glicyne max*, since it might be an important point to consider in the management of crop rotation in the Southern Brazil.

The genus *Curvularia* is considered a species complex, due to the usual overlap in morphology of different species, and to an accurate identification molecular data based on ITS, *gapdh* and *tef1* loci, are needed (Manamgoda et al. 2014, 2015; Marin-Felix et al. 2017; Tan et al. 2018). Morphology of *Curvularia* sp. nov. did not coincide with any previously reported species. *Curvularia intermedia*, described on to *L. multiflorum* from china, (Li et al. 2019), could be compared with the Brazilian species, however, in the present analysis, the former clusters far distant from the latter (Fig. 8)

The genus *Exserohilum* is morphologically different from *Bipolaris, Curvularia* and *Pyrenophora* by the formation of a protruding hilum (Leonard and Suggs 1974). Currently, a total of 11 species are accepted for *Exserohilum*, based on a combined analysis of 9 nuclear loci (Hernández-Restrepo et al. 2018). *Exserohilum rostratum* was first described in *Eragrostis major* by Drechsler in 1923 (Sivanesan 1987), but nowadays, it is known to be a cosmopolitan species, attacking plants from all over the world (Farr & Rossman 2020), such as many classes of grasses, including Poaceae (Brunings et al. 2009). In Brazil this pathogen has been reported in *Brachiaria ruziziensis*, *Oryza sativa*, *Sorghum* sp., *Sorghum bicolor, Triticum aestivum*, *Zea mays*, and now, on *L. multiflorum* (Farr and Rossman 2020).

The genus *Pyrenophora* (asexual morph, *Drechslera*), comprises several saprophytic and pathogenic species related to Poaceae (Marin-Felix et al. 2019). *Pyrenophora nobleae* was introduced in 1977, and isolated from yellowish leaves and seeds of *Lolium* sp. in New Zealand (Lam 1984). The last report of this species, however, was done more than 30 years ago, based only on morphological observations (Pennycook 1989). Morphology of *Pyrenophora* often overlaps those of *Bipolaris* and *Curvularia* (Marin-Felix et al. 2017), and thus far should not be used solely to define species boundaries (Marin-Felix et al. 2019). In this sense, the phylogenetic survey presented here (Fig. 12) places de Brazilian isolate irrefutably as *P. nobleae*.

To date, 54 genera of species within Poaceae have been confirmed as hosts of *Pyricularia* (Zheng et al. 2018). Among these, *P. oryzae* is the pathogen responsible for the most important diseases such as rice, wheat, and millet (Klaubauf et al. 2014). Furthermore, the sexual morph of *P. oryzae* infecting *L. multiflorum* can induce spike disease on *Triticum aestivum* (Farman et al. 2017). Thus, crop rotation of such crops with *L. multiflorum* should be used with caution, as stated for *B. sorokiniana*.

In summary, the present survey identified and demonstrated the pathogenicity of seven fungal species causing leaf spots on *L. multiflorum* from Southern Brazil, being two new host-associations (*E. rostratum* and *C. plurivorum*), three new records from Brazil (*Bipolaris sorokiniana, P. nobleae*, and *P. oryzae*), and two proposed as new species *Cercospora* sp. nov and *Curvularia* sp. nov).

Before the present study, six fungal species were known related *L. multiflorum* from Brazil, being three of *Fusarium* (*F. asiaticum*, *F. cortaderiae*, and *F. graminearum*) and three of *Puccinia* (*P. coronata*, *P. graminis* subsp. *graminis*, and *P. hordei*) (Farr & Rossman, 2020).

The results of the present study brings fundamental basis for identifying, in the future, can help to design control strategies for them in Southern grazing areas of Brazil and other countries.

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TABLES

Table 1. Primers and PCR program conditions used in this study for amplification and sequencing

Loci[1]	Primer	Direction	Sequence (5'–3')	Primer reference	PRC conditions					Program reference
					Initial den.	Dena.	Align.	Exten.	Final exten.	
act	ACT-512F	Foward	ATGTGCAAGGCC GGTTTCGC	Carbone & Kohn 1999	95 °C, 4 m	95 °C, 30 s 35 cycles	58 °C, 30 s	72 °C, 45 s	72 °C, 7 m	Weir et al. 2012
	ACT-783R	Reverse	TACGAGTCCTTC TGGCCCAT							
cmdA	CAL-228F	Foward	GAGTTCAAGGA GGCCTTCTCCC	Carbone & Kohn 2000	94 °C, 5 m	94 °C, 30 s, 40 cycles	58 °C, 30 s	72 °C, 30 s	72 °C, 7 m	Groenewal et al., 2013
	CAL-2Rd	Reverse	TGRTCNGCCTCD CGGATCATCTC	Groenewald et al., 2013						
LSU	LR0R	Foward	ACCCGCTGAACT TAAGC	Vilgalys and Hester, 1990	95 °C, 3m	94 °C, 40 s, 30 cycles	52 °C, 40 s	72 °C, 1 m	72 °C, 10 m	Mandagoda et al. 2012
	LR5	Reverse	TCCTGAGGGAA ACTTCG	Brown et al., 2014						
gapdh	gpd1	Foward	CAACGGCTTCGG TCGCATTG	Berbee et al., 1999	96 °C, 2m	96 °C, 1 m, 35 cycles	52 °C, 1 m	72 °C, 45 s	72 °C, 10 m	Mandagoda et al. 2012
	gpd2	Reverse	GCCAAGCAGTT GGTTGTGC							
ITS	ITS-1F	Foward	CTT GGT CAT TTA GAG GAA GTA A	Gardes & Bruns 1993	94 °C, 3m	94 °C, 1 m, 30 cycles	55 °C, 1 m (ITSF) 57 °C,	72 °C, 45 s	72 °C, 10 m	White et al. 1990
	ITS-1	Foward	TCC GTA GGT GAA CCT GCG G	White et al. 1990						

	ITS-4	Reverse	TCC TCC GCT TAT TGA TAT GC				1 m (ITS1)			
tef1	EF983F	Foward	GCYCCYGGHCA YCGTGAYTTYAT	Schoch et al., 2009	95 °C, 3m	94 °C, 40 s, 30 cycles	54 °C, 50 s	72 °C, 1 m	72 °C, 10 m	Mandagoda et al. 2012
	EF2218 R	Reverse	ATGACACCRACR GCRACRGTYTG							
h3	CYLH3 F	Foward	AGGTCCACTGGT GGCAAG	Crous et al., 2004	95 °C, 5m	95 °C, 30 s, 40 cycles	52 °C, 30 s	72 °C, 45 s	72 °C, 5 m	Bakhshi et al. 2015
	CYLH3 R	Reverse	AGCTGGATGTCC TTGGACTG							

[1]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *act*: actin; *cmdA*: calmodulin; *his3*: histone; *gapdh*: partial glyceraldehyde-3-phosphate dehydrogenase gene; *tef1*: partial translation elongation factor 1-α.

Table 2. Details of *Bipolaris* strains subjected to multi-gene DNA sequence analysis.

Species	Strain no.[1]	Host	Location	GenBank accession numbers[2]			Reference
				ITS	*gapdh*	*tef1*	
Bipolaris bicolor	CBS 690.96	—	Cuba	KJ909762	KM042893	KM093776	Manamgoda et al. 2014
B. chloridis	CBS 242.77	*Chloris gayana*	Australia	JN192372	JN600961	—	Manamgoda et al. 2011
B. coffeana	C 12.04	*Cynodon dactylon*	USA	KM230385	KM034837	KM093781	Manamgoda et al. 2014
	MFLUCC 12-MFU0088	*Digitaria* sp.	Thailand	KJ922385	KM034841	KM093784	Manamgoda et al. 2014
	M 1129 MUS002	*Bouteloua gracilis*	USA	KJ922384	KM034836	KM093780	Manamgoda et al. 2014
	M 1130 MUS003	*Bouteloua gracilis*	USA	KM230387	KM034835	KM093779	Manamgoda et al. 2014

	MFU0090	Poaceae	Thailand	KM230386	KM034840	KM093783	Manamgoda et al. 2014
	ICMP 6128	*Cynodon dactylon*	New Zealand	JX256412	KM034839	JX266581	Manamgoda et al. 2011; 2014
B. cookei	AR 5185	*Sorghum* sp.	Japan	KJ922391	KM034833	KM093777	Manamgoda et al. 2014
	MAFF 51191	*Sorghum bicolor*	Japan	KJ922392	KM034834	KM093778	Manamgoda et al. 2014
B. cynodontis	CBS 109894	*Cynodon dactylon*	Hungary	KJ909767	KM034838	KM093782	Manamgoda et al. 2014
B. drechsleri	AR 4841; CBS 136207	*Microstegium vimineum*	USA	KF500530	KF500533	KM093760	Crous et al. 2013; Manamgoda et al. 2014
	MUS 0028	*Microstegium vimineum*	USA	KF500532	KF500535	KM093761	Crous et al. 2013; Manamgoda et al. 2014
	FIP 373	Ornamental	USA	KF500531	KF500534	KM093759	Crous et al. 2013
B. crotonis (= *B. eleusines*)	CBS 274.91	*Eleusine indica*	Australia	KJ909768	KM034820	KM093758	Berbee et al. 1999
B. heveae	CBS 241.92	*Hevea* sp.	Nigeria	KJ909763	KM034843	KM093791	Manamgoda et al. 2014
B. maydis	CBS 137271/C5	*Zea mays*	USA	AF071325	KM034846	KM093794	Berbee et al. 1999; Manamgoda et al. 2014
	AR 5182	*Sorghum bicolor*	Japan	KM230388	KM034844	KM093792	Manamgoda et al. 2014
	AR 5183	*Sorghum bicolor*	Japan	KM230390	KM034848	KM093796	Manamgoda et al. 2014
	M 1122/ C4	*Zea mays*	USA	KM230389	KM034847	KM093795	Manamgoda et al. 2014
	CBS 136.29	*Zea mays*	Japan	KJ909769	KM034845	KM093793	Manamgoda et al. 2014
B. microlaenae	BRIP 15613	*Microlaena stipoides*	Australia	JN601032	JN600974	JN601017	Manamgoda et al. 2011
B. microstegii	AR 4840; CBS 132550	*Microlaena vimineum*	USA	JX089579	JX089575	KM093756	Manamgoda et al. 2014

	AR 5192	*Microlaena vimineum*	USA	KM230391	KM034819	KM093757	Manamgoda et al. 2014
B. oryzae	MFLUCC 100715	*Oryza sativa*	Thailand	JX256416	JX276430	JX266585	Manamgoda et al. 2012
	MFLUCC 100733	*Oryza sativa*	Thailand	JX256417	KM042898	KM093790	Manamgoda et al. 2012
	MAFF 235499	*Oryza sativa*	Japan	KJ922383	KM042897	KM093789	Manamgoda et al. 2014
	AR3797	*Panicum virgatum*	USA	KM230392	KM042894	KM093786	Manamgoda et al. 2014
	AR 5204	*Panicum virgatum*	USA	KM230393	KM042895	KM093787	Manamgoda et al. 2014
B. panici-miliacei	CBS 199.29	*Panicum miliaceum*	Japan	KJ909773	KM042896	KM093788	Manamgoda et al. 2014
B. peregianensis	DAOM 221998	*Cynodon dactylon*	Australia	KJ922393	KM034849	KM093797	Manamgoda et al. 2014
	BRIP 12790	*Cynodon dactylon*	Australia	JN601034	JN600977	JN601022	Manamgoda et al. 2011
B. sacchari	ICMP 6227	*Oplismenus imbecillis*	New Zealand	KJ922386	KM034842	KM093785	Manamgoda et al. 2014
B. salviniae	IMI 228224	*Salvinia auriculata*	Brazil	KJ922390	KM034829	KM093772	Manamgoda et al. 2014
B. salviniae (= *B. melinidis*)	BRIP 12898	*Melinis minutiflora*	Australia	JN601035	JN600972	KM093771	Manamgoda et al. 2011
B. sorokiniana (= *B. multiformis*)	CBS 120.24	—	Italy	KJ909776	KM034821	KM093762	Manamgoda et al. 2014
	CBS 110.14	*Hordeum* sp.	USA	KJ922381	KM034822	KM093763	Manamgoda et al. 2014
	LIPP BI4	*Lolium multiflorum*	Brazil	**To be assigned**			This study
	FIP 499	*Phalaris arundinaceae*	USA	KJ922382	KM034828	KM093769	Manamgoda et al. 2014
	MAFF 236448	*Zea mays*	Japan	KJ909792	KM034826	KM093767	Manamgoda et al. 2014

	MAFF 235500	*Paddy field soil*	Japan	KJ909789	KM034823	KM093764	Manamgoda et al. 2014
	MAFF 235501	*Zea mays*	Japan	KJ909791	KM034825	KM093766	Manamgoda et al. 2014
	MAFF 238877	*Hordeum vulgare*	Japan	KJ909790	KM034824	KM093765	Manamgoda et al. 2014
	CBS 480.74	*Tribulus terrestris*	South Africa	KJ909771	KM034827	KM093768	Manamgoda et al. 2014
B. urochloae	ATCC 58317	*Urochloa panicoides*	Australia	KJ922389	KM230396	KM093770	Manamgoda et al. 2014
B. victoriae	CBS 327.64	*Avena sativa*	USA	KJ909778	KM034811	KM093748	Manamgoda et al. 2014
	DAOM 147449	*Avena sativa*	USA	KJ909785	KM034812	KM093749	Manamgoda et al. 2014
B. yamadae	DAOM 147441	*Saccharum officinarum*	Cuba	KJ922388	KM034831	KM093774	Manamgoda et al. 2014
	MAFF 235507	*Zea mays*	Japan	KJ922387	KM034832	KM093775	Manamgoda et al. 2014
	CBS 202.29	*Panicum miliaceum*	Japan	KJ909779	KM034830	KM093773	Manamgoda et al. 2014
B. zeae	AR 3795	*Panicum virgatum*	USA	KJ909786	KM034816	KM093753	Manamgoda et al. 2014
	AR 5181	*Sorghum bicolor*	Japan	KM230394	KM034817	KM093754	Manamgoda et al. 2014
	DAOM 211267	*Triticum* sp.	Canada	KJ909787	KM034818	KM093755	Manamgoda et al. 2014
B. zeicola	AR 5166	*Sorghum* sp.	USA	KJ909788	KM034813	KM093750	Manamgoda et al. 2014
	AR 5168	*Sorghum* sp.	USA	KM230397	KM034814	KM093751	Manamgoda et al. 2014
	FIP 532	*Zea mays*	USA	KM230398	KM034815	KM093752	Manamgoda et al. 2014
Curvularia lunata	CBS 157.34	unknown	Indonesia	JX256430	JX276442	JX266597	Manamgoda et al. 2012

[1]BRIP: Herbarium of Plant Pathology, Department of Primary Industries; ATCC: American Type Culture Collection, Virginia, United States; AR, FIP, MFU, MUS: Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, Maryland. MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; CBS: CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; E.G.S .: Collection of E.G. Simmons ICMP = PDDCC: International Collection of Plant Microorganisms, Landcare Research, Private Bag 92170, Auckland, New Zealand;

IMI: International Institute of Mycology, CABI-Bioscience Egham, Bakeham Lane, U.K.; MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil.

[2]ITS: internal transcribed spacers intervening 5.8S nrDNA; *gapdh*: partial glyceraldehyde-3-phosphate dehydrogenase gene; *tef1*: partial translation elongation factor 1-α

Table 3. Details of *Cercospora* strains subjected to multi-gene DNA sequence analysis.

				GenBank accession numbers[2]				
Species	Strain no.[1]	Host	Location	ITS	*act*	cmdA	his3	Reference
Cercospora althaeina	CCTU 1001	*Althaea rosea*	Iran	KJ886392	KJ885909	KJ885748	KJ886070	Bakhshi et al. 2015
	CCTU 1026	*Althaea rosea*	Iran	KJ886393	KJ885910	KJ885749	KJ886071	Bakhshi et al. 2015
C. apii	CCTU 1069	*Cynanchum acutum*	Iran	KJ886410	KJ885927	KJ885766	KJ886088	Bakhshi et al. 2015
	CCTU 1215	*Cynanchum acutum*	Iran	KJ886412	KJ885929	KJ885768	KJ886090	Bakhshi et al. 2015
C. armoraciae	CBS 250.67; CPC 5088[T]	*Armoracia rusticana*	Romania	JX143545	JX143053	JX142807	JX142561	Groenewald et al. 2013
C. beticola	CCTU 1065	*Chenopodium* sp.	Iran	KJ886425	KJ885942	KJ885781	KJ886102	Bakhshi et al. 2015
	CCTU 1087	*Chenopodium* sp.	Iran	KJ886427	KJ885944	KJ885783	KJ886103	Bakhshi et al. 2015

C. bizzozeriana	CBS 258.67; CPC 5061^{T}	*Cardaria draba*	Romania	JX143546	JX143054	JX142808	JX142562	Groenewald et al. 2013
	CBS 540.71; IMI 161110; CPC 5060	*Cardaria draba*	Romania	JX143548	JX143056	JX142810	JX142564	Groenewald et al. 2013
C. chenopodii	CCTU 1060; IRAN 2652C	*Chenopodium album*	Iran	KJ886438	KJ885955	KJ885794	KJ886116	Bakhshi et al. 2015
	CCTU 1163	*Chenopodium album*	Iran	KJ886440	KJ885957	KJ885796	KJ886118	Bakhshi et al. 2015
	CCTU 1033	*Chenopodium album*	Iran	KJ886437	KJ885954	KJ885793	KJ886115	Bakhshi et al. 2015
C. convolvulicola	CCTU 1083; CBS 136126^{T}	*Convolvulus arvensis*	Iran	KJ886441	KJ885958	KJ885797	KJ886119	Bakhshi et al. 2015
	CCTU 1083.2	*Convolvulus arvensis*	Iran	KJ886442	KJ885959	KJ885798	KJ886120	Bakhshi et al. 2015
C. cylindracea	CCTU 1016	*Cichorium intybus*	Iran	KJ886446	KJ885963	KJ885802	KJ886124	Bakhshi et al. 2015
	CCTU 1044; CBS 136021	*Lactuca serriola*	Iran	KJ886447	KJ885964	KJ885803	KJ886125	Bakhshi et al. 2015
C. cf. *flagellaris*	CCTU 1007; CBS 136031	*Hydrangea* sp.	Iran	KJ886456	KJ885973	KJ885812	KJ886134	Bakhshi et al. 2015

	CCTU 1027; CBS 136034	*Lepidium sativum*	Iran	KJ886459	KJ885976	KJ885815	KJ886137	Bakhshi et al. 2015
C. gamsiana	CCTU 1205; CBS 136127; IRAN 2677C	*Sesamum indicum*	Iran	KJ886435	KJ885952	KJ885791	KJ886113	Bakhshi et al. 2015
	CCTU 1208; IRAN 2678C	*Sonchus* sp.	Iran	KJ886436	KJ885953	KJ885792	KJ886114	Bakhshi et al. 2015
C. cf. *gossypii*	CCTU 1070; CBS 136137	*Gossypium herbaceum*	Iran	KJ886467	KJ885984	KJ885823	KJ886145	Bakhshi et al. 2015
	CCTU 1055; IRAN 2650C	*Hibiscus trionum*	Iran	KJ886463	KJ885980	KJ885819	KJ886141	Bakhshi et al. 2015
C. plantaginis	CCTU 1095	*Plantago lanceolata*	Iran	KJ886403	KJ885920	KJ885759	KJ886081	Bakhshi et al. 2015
	CCTU 1041; CPC 24910	*Plantago lanceolata*	Iran	KJ886400	KJ885917	KJ885756	KJ886078	Bakhshi et al. 2015
C. pseudochenopodii	CCTU 1176	*Chenopodium album*	Iran	KJ886518	KJ886035	KJ885874	KJ886196	Bakhshi et al. 2015
	CCTU 1045	*Chenopodium* sp.	Iran	KJ886517	KJ886034	KJ885873	KJ886195	Bakhshi et al. 2015
	CCTU 1173; CBS	*Chenopodium* sp.	Iran	Kj886516	KJ886033	KJ885872	KJ886194	Bakhshi et al. 2015

	136448; IRAN 2672C (TYPE)							
C. rumicis	CCTU 1123	*Rumex crispus*	Iran	KJ886521	KJ886038	KJ885877	KJ886199	Bakhshi et al. 2015
	CCTU 1129; IRAN 2662C	*Rumex crispus*	Iran	KJ886522	KJ886039	KJ885878	KJ886200	Bakhshi et al. 2015
	CCTU 1121	*Urtica dioica*	Iran	KJ886520	KJ886037	KJ885876	KJ886198	Bakhshi et al. 2015
Cercospora sp. G	CCTU 1197	*Bidens tripartita*	Iran	KJ886540	KJ886057	KJ885896	KJ886218	Bakhshi et al. 2015
	CCTU 1015; CBS 136024; IRAN 2645C	*Plantago major*	Iran	KJ886528	KJ886045	KJ885884	KJ886206	Bakhshi et al. 2015
	CCTU 1058	*Helminthotheca echioides*	Iran	KJ886534	KJ886051	KJ885890	KJ886212	Bakhshi et al. 2015
	CCTU 1090	*Abutilon theophrasti*	Iran	KJ886536	KJ886053	KJ885892	KJ886214	Bakhshi et al. 2015
	CCTU 1079; CBS 136025	*Amaranthus retroflexus*	Iran	KJ886535	KJ886052	KJ885891	KJ886213	Bakhshi et al. 2015
	CCTU 1054	Amaranthus sp.	Iran	KJ886533	KJ886050	KJ885889	KJ886211	Bakhshi et al. 2015

	CCTU 1122	Amaranthus sp.	Iran	KJ886538	KJ886055	KJ885894	KJ886216	Bakhshi et al. 2015
	CBS 115518; CPC 5360	*Bidens frondosa*	New Zealand	JX143681	JX143195	JX142949	JX142703	Groenewald et al. 2013
	CCTU 1002	*Celosia cristata*	Iran	KJ886527	KJ886044	KJ885883	KJ886205	Bakhshi et al. 2015
	CCTU 1144; CBS 136130	*Cucurbita maxima*	Iran	KJ886539	KJ886056	KJ885895	KJ886217	Bakhshi et al. 2015
	CCTU 1046	*Plantago major*	Iran	KJ886531	KJ886048	KJ885887	KJ886209	Bakhshi et al. 2015
	CCTU 1116	*Plantago major*	Iran	KJ886537	KJ886054	KJ885893	KJ886215	Bakhshi et al. 2015
	CCTU 1020; CBS 136023	*Sorghum halepense*	Iran	KJ886529	KJ886046	KJ885885	KJ886207	Bakhshi et al. 2015
C. uwebrauniana	CCTU 1200; CBS 138581^{T}	*Heliotropium europaeum*	Iran	KJ886408	KJ885925	KJ885764	KJ886086	Bakhshi et al. 2015
	CCTU 1134	*Heliotropium europaeum*	Iran	KJ886407	KJ885924	KJ885763	KJ886085	Bakhshi et al. 2015
C. violae	CCTU 1025; IRAN 2646C	*Viola* sp.	Iran	KJ886543	KJ886060	KJ885899	KJ886221	Bakhshi et al. 2015

	CBS 251.67; CPC 5079^{T}	*Viola tricolor*	Romania	JX143737	JX143250	JX143004	JX142758	Groenewald et al. 2013
C. zebrina	CCTU 1181	*Trifolium repens*	Iran	KJ886548	KJ886065	KJ885904	KJ886226	Bakhshi et al. 2015
	CCTU 1185	*Vicia* sp.	Iran	KJ886549	KJ886066	KJ885905	KJ886391	Bakhshi et al. 2015
C. sorghicola	CCTU 1173; CBS 136448; IRAN 2672C^{T}	*Sorghum halepense*	Iran	KJ886525	KJ886042	KJ885881	KJ886203	Bakhshi et al. 2015
Cercospora sp. nov.	C2A LIPP^{T}	*Lolium multiflorum*	Brazil	**To be assigned**				This study

[1]CCTU: Culture Collection of Tabriz University, Tabriz, Iran; CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; CPC: IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Tehran, Iran; Culture collection of Pedro Crous, housed at CBS; IMI: International Institute of Mycology, CABI-Bioscience Egham, Bakeham Lane, U.K.; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil. T: ex-type cultures.

[2]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *act*: actin; *cmdA*: calmodulin; *his3*: histone.

Table 4. Details of *Colletotrichum* strains subjected to multi-gene DNA sequence analysis.

				GenBank accession numbers[2]			
Species	Strain no.[1]	Host	Location	ITS	*his3*	*Act*	Reference
Colletotrichum brevisporum	CBS 129957	*Anthurium* sp.	Thailand	MG600762	MG600908	MG600966	Damm et al. 2018
	CBS 129958	*Anthurium* sp.	Thailand	MG600763	MG600909	MG600967	Damm et al. 2018
	CBS 512.75	*Carica papaya*	Australia	MG600761	MG600907	MG600965	Damm et al. 2018

C. cacao	CBS 119297[ET]	*Theobroma cacao*	Costa Rica	MG600772	MG600916	MG600976	Damm et al. 2018
C. cattleyicola	CBS 170.49[ET]	*Cattleya* sp.	Belgium	MG600758	MG600905	MG600963	Damm et al. 2018
C. cliviicola	CBS 125375 [ET]	*Clivia miniata*	China	MG600733	MG600892	MG600939	Damm et al. 2018
	CBS 133705	*Clivia* sp.	South Africa	MG600732	MG600891	MG600938	Damm et al. 2018
C. gloeosporioides	CBS 112999 [ET]	*Citrus sinensis*	Italy	JQ005152	JQ005413	JQ005500	Damm et al. 2012
C. magnum	CBS 519.97 [ET]	*Citrullus lanatus*	USA	MG600769	MG600913	MG600973	Damm et al. 2018
	IMI 391662	*Citrullus lanatus*	USA	MG600771	MG600915	MG600975	Damm et al. 2018
	CBS 575.97	*Citrullus lanatus*	USA	MG600770	MG600914	MG600974	Damm et al. 2018
C. musicola	CBS 132885 [ET]	*Musa* sp.	Mexico	MG600736	MG600895	MG600942	Damm et al. 2018
	CBS 127557	*Musa* sp.	Mexico	MG600737	MG600896	MG600943	Damm et al. 2018
C. orchidearum	CBS 135131 [ET]	*Dendrobium nobile*	Netherlands	MG600738	MG600897	MG600944	Damm et al. 2018
	CBS 136877	*Dendrobium nobile*	Netherlands	MG600739	MG600898	MG600945	Damm et al. 2018
C. piperis	IMI 71397, CPC 21195 [ET]	*Piper nigrum*	Malaysia	MG600760	MG600906	MG600964	Damm et al. 2018
C. plurivorum	CBS 125474 [ET]	*Coffea* sp.	Vietnam	MG600718	MG600887	MG600925	Damm et al. 2018
	CBS 125473	*Coffea* sp.	Vietnam	MG600717	MG600886	MG600924	Damm et al. 2018
	LFN0008	*Glycine max.*	Brazil	KT696336	KT696311	KT696275	Damm et al. 2019

	CBS 132443	*Gossypium* sp.	Brazil	MG600719	MG600888	MG600926	Damm et al. 2018
	LIPP COLL1B	*Lolium multiflorum*	Brazil	**To be assigned**			This study
	CBS 132444	*Gossypium* sp.	Brazil	MG600720	MG600889	MG600927	Damm et al. 2018
	CBS 903.69	*Phaseolus lunatus*	Benin	MG600721	MG600890	MG600928	Damm et al. 2018
C. sojae	LFN0009	*Glycine max*	Brazil	KT696354	KT696318	KT696281	Damm et al. 2019
	ATCC 62257 ET	*Glycine max*	USA	MG600749	MG600899	MG600954	Damm et al. 2018
C. vittalense	CBS 126.25	Orchid	unknown	MG600735	MG600894	MG600941	Damm et al. 2018
	CBS 181.82	*Theobroma cacao*	India	MG600734	MG600893	MG600940	Damm et al. 2018

[1]CBS-KNAW Fungal Biodiversity Center, Utrecht, The Netherlands; IMI: International Institute of Mycology, CABI-Bioscience Egham, Bakeham Lane, U.K.; CPC: Culture collection of Pedro Crous, housed at CBS; ATCC: American Type Culture Collection, Virginia, USA; BCC: BIOTEC culture collection, Bangkok, Thailand; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil. ET: Ex-holotype or ex-epitype cultures

[2]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *his3*: histone; *act*: actin.

Table 5. Details of *Curvularia* strains subjected to multi-gene DNA sequence analysis.

				GenBank accession numbers[2]			
Species	Strain no.[1]	Host	Location	ITS	*gapdh*	*tef1*	Reference
Bipolaris maydis	CBS 136.29	*Zea mays*	USA	AF071325	KM034846	KM093794	Berbee et al. 1998
Curvularia affinis	CBS 154.34	Unknown	Indonesia	KJ909780	KM230401	KM196566	Manamgoda et al. 2014
C. akaii	CBS 317.86	Unknown	Japan	KJ909782	KM230402	KM196569	Manamgoda et al. 2014
C. akaiiensis	BRIP 16080	Unknown	India	KJ415539	KJ415407	KJ415453	Tan et al. 2014

C. alcornii	MFLUCC 10-0703	*Zea mays*	Thailand	JX256420	JX276433	JX266589	Manamgoda et al. 2012
C. asianensis	MFLUCC 10-0711	*Panicum* sp.	Thailand	JX256424	JX276436	JX266593	Manamgoda et al. 2012
C. australiensis	BRIP 12044	*Oryza sativa*	Australia	KJ415540	KJ415406	KJ415452	Tan et al. 2014
C. australis	BRIP 12521	*Sporobolus caroli*	Australia	KJ415541	KJ415405	KJ415451	Tan et al. 2014
C. beasleyi	BRIP 10972	*Chloris gayana*	Australia	MH414892	MH433638	MH433654	Tan et al. 2018
C. beasleyi	BRIP 15854	*Leersia hexandra*	Australia	MH414893	MH433639	MH433655	Tan et al. 2018
C. beerburrumensis	BRIP 12942	*Eragrostis bahiensis*	Australia	MH414894	MH433634	MH433657	Tan et al. 2018
C. beerburrumensis	BRIP 12555	*Eragrostis sororia*	Australia	MH414895	MH433640	MH433656	Tan et al. 2018
C. borreriae	MFLUCC 11-0422	Unknown Poaceae	Thailand	KP400638	KP419987	KM196571	Manamgoda et al. 2015
C. bothriochloae	BRIP 12522	*Bothriochloa bladhii*	Australia	KJ415543	KJ415403	KJ415449	Tan et al. 2014
C. brachyspora	CBS 186.50	Soil	India	KJ922372	KM061784	KM230405	Manamgoda et al. 2014
C. buchloës	CBS 246.49	*Buchloë dactyloides*	USA	KJ909765	KM061789	KM196588	Manamgoda et al. 2014
C. chiangmaiensis	CPC 28829	*Zea mays*	Thailand	MF490814	MF490836	MF490857	Marin et al. 2017
C. coatesiae	BRIP 24170	Air	Australia	MH414896	MH433635	MH433658	Tan et al. 2018
C. coatesiae	BRIP 24261	*Litchi chinensis*	Australia	MH414897	MH433636	MH433659	Tan et al. 2018
C. crustacea	BRIP 13524	*Sporobolus* sp.	Indonesia	KJ415544	KJ415402	KJ415448	Tan et al. 2014
C. dactyloctenicola	CPC 28810	*Dactyloctenium aegyptium*	Thailand	MF490815	MF490837	MF490858	Marin and Crous 2017
C. dactyloctenii	BRIP 12846	*Dactyloctenium radulans*	Australia	KJ415545	KJ415401	KJ415447	Tan et al. 2014

C. ellisii	CBS 193.62	Air	Pakistan	JN192375	JN600963	JN601007	Manamgoda et al. 2011
C. geniculata	CBS 187.50	*Andropogon sorghum*	Indonesia	KJ909781	KM083609	KM230410	Manamgoda et al. 2014
C. harveyi	BRIP 57412	*Triticum aestivum*	Australia	KJ415546	KJ415400	KJ415446	Tan et al. 2014
C. hawaiiensis	BRIP 11987	*Oryza sativa*	USA	KJ415547	KJ415399	KJ415445	Tan et al. 2014
C. heteropogonis	CBS 284.91	*Heteropogon contortus*	Australia	KJ415549	JN600969	JN601013	Tan et al. 2014
C. hominis	AR 5118	*Lolium perene*	USA	KP400639	KP419988	KM196580	Manamgoda et al. 2015
C. homomorpha	CBS 156.60	Air	USA	JN192380	JN600970	JN601014	Manamgoda et al. 2011
C. inaequalis	CBS 102.42	Soil	France	KJ922375	KM061787	KM196574	Manamgoda et al. 2014
C. intermedia	SDHM20^{T}	*Lolium multiflorum*	China	MH118553	–	–	Li et al 2019
C. kenpeggii	BRIP 14530	*Triticum aestivum*	Australia	MH414900	MH433644	MH433662	Tan et al. 2018
C. mebaldsii	BRIP 12900	*Cynodon transvaalensis*	Australia	MH414902	MH433647	MH433664	Tan et al. 2018
C. mebaldsii	BRIP 13983	*Cynodondactylon transvaalensis*	Australia	MH414903	MH433646	MH433665	Tan et al. 2018
C. miyakei	CBS 197.29	*Eragrostis pilosa*	Japan	KJ909770	KM083611	KM196568	Manamgoda et al. 2014
C. muehlenbeckiae	CBS 144.63	*Sorghum* sp.	USA	KP400647	KP419996	KM196578	Manamgoda et al. 2015
C. neergaardii	BRIP 12919	*Oryza sativa*	Ghana	KJ415550	KJ415397	KJ415443	Tan et al. 2014
C. nodosa	CPC 28800	*Digitaria ciliaris*	Thailand	MF490816	MF490838	MF490859	Marin et al. 2017
C. ovariicola	CBS 470.90	*Eragrostis interrupta*	Australia	JN192384	JN600976	JN601020	Manamgoda et al. 2011
C. pallescens	CBS 156.35	Air	Indonesia	KJ922380	KM083606	KM196570	Manamgoda et al. 2014
C. perotidis	CBS 350.90	*Perotis rara*	Australia	JN192385	KJ415394	JN601021	Manamgoda et al. 2011

C. petersonii	BRIP 14642	*Dactyloctenium aegyptium*	Australia	MH414905	MH433650	MH433668	Tan et al. 2018
C. pisi	CBS 190.48	*Pisum sativum*	Canada	KY905678	KY905690	KY905697	Marin et al. 2017
C. platzii	BRIP 27703b	*Cenchrus clandestinus*	Australia	MH414906	MH433651	MH433669	Tan et al. 2018
C. prasadii	CBS 143.64	*Jasminum sambac*	India	KJ922373	KM061785	KM230408	Manamgoda et al. 2014
C. protuberata	CBS 376.65	*Deschampsia flexuosa*	UK	KJ922376	KM083605	KM196576	Manamgoda et al. 2014
C. pseudobrachyspora	CPC 28808	*Eleusine indica*	Thailand	MF490819	MF490841	MF490862	Marin et al. 2017
C. ravenelii	BRIP 13165	*Sporobolus fertilis*	Australia	JN192386	JN600978	JN601024	Manamgoda et al. 2011
C. richardiae	BRIP 4371	*Richardia brasiliensis*	Australia	KJ415555	KJ415391	KJ415438	Tan et al. 2014
C. robusta	CBS 624.68	*Dichanthium annulatum*	USA	KJ909783	KM083613	KM196577	Manamgoda et al. 2014
C. ryleyi	BRIP 12554	*Sporobolus creber*	Australia	KJ415556	KJ415390	KJ415437	Tan et al. 2014
C. soli	CBS 222.96	Soil	Papua New Guinea	KY905679	KY905691	KY905698	Marin et al. 2017
C. sorghina	BRIP 15900	*Sorghum bicolor*	Australia	KJ415558	KJ415388	KJ415435	Tan et al. 2014
Curvularia sp.	AR 5117	*Lolium perene*	USA	KP400655	KP645349	KP735698	Manamgoda et al. 2016
Curvularia sp. nov.	LIPP CU1^{T}	*Lolium multiflorum*	Brazil	**To be assigned**			This study
C. spicifera	CBS 274.52	Soil	Spain	JN192387	JN600979	JN601023	Manamgoda et al. 2011
C. subpapendorfii	CBS 656.74	Soil	Egypt	KJ909777	KM061791	KM196585	Manamgoda et al. 2014
C. tripogonis	BRIP 12375	*Tripogon loliiformis*	Australia	JN192388	JN600980	JN601025	Manamgoda et al. 2011

C. tropicalis	BRIP 14834	*Coffea arabica*	India	KJ415559	KJ415387	KJ415434	Tan et al. 2014
C. tsudae	ATCC 44764	*Chloris gayana*	Japan	KC424596	KC747745	KC503940	Deng et al. 2012
C. variabilis	CPC 28815	*Chloris barbata*	Thailand	MF490822	MF490844	MF490865	Marin et al. 2017
C. verruculosa	CBS 150.63	*Punica granatum*	India	KP400652	KP645346	KP735695	Manamgoda et al. 2015
C. warraberensis	BRIP 14817	*Dactyloctenium aegyptium*	Australia	MH414909	MH433653	MH433672	Tan et al. 2018

[1]CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; Note: AR: Isolates housed in Systematic Mycology and Microbiology Laboratory, United States Department of Agriculture, Agricultural Research Service, Beltsville, Maryland. MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; ATCC: American Type Culture Collection, Virginia, USA; CPC: Culture collection of Pedro Crous, housed at CBS; SDHM: Department of Weed Science, Institute of Plant Protection, Shandong Academy of Agricultural Sciences, China; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil.

[2]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *tef1*: partial translation elongation factor 1-α.

Table 6. Details of *Exserohilum* strains subjected to multi-gene DNA sequence analysis.

Species	Strain no.[1]	Host	Location	GenBank accession numbers[2]		Reference
				ITS	*gapdh*	
Curvularia heteropogonicola	CBS 128052; BRIP 14579^{T}	*Heteropogon contortus*	India	KJ415548	KJ415398	Tan et al. 2014
Exserohilum holmii	CBS 413.65^{IST} of *H. holmii*	*Dactyloctenium aegyptium*	USA	LT837459	LT715890	Restrepo et al. 2018
	CBS 505.90; IMI 281326^{IT}; CBS 132712^{T} of *E. curvatum*	*Sorghum vulgare*	Venezuela	KT265252	LT715889	Restrepo et al. 2018

	CBS 128053	*Dactyloctenium aegyptium*	Thailand	KT265253	LT882555	Restrepo et al. 2018
E. minor	BRIP 14614	*Dactyloctenium aegyptium*	Australia	LT837468	LT715885	Restrepo et al. 2018
	BRIP 14615; IMI 294530b^{IT}; DAR 51591; ATCC62323^{T} of *S. minor*	*Dactyloctenium aegyptium*	Australia	LT837469	LT883544	Restrepo et al. 2018
E. monoceras	BRIP 12236	*Echinochloa colona*	Australia	LT837472	LT715876	Restrepo et al. 2018
	BRIP 12271; ATCC 36561; ATCC 36562^{A} of *S. monoceras*	*Echinochloa colona*	Australia	LT837475	LT883548	Restrepo et al. 2018
E. neoregeliae	CBS 132832; IM201-D^{T}	*Neoregelia carolinae*	Japan	LT837476	LT715886	Restrepo et al. 2018
	CBS 132833	*Neoregelia carolinae*	Japan	LT837477	LT715887	Restrepo et al. 2018
E. oryzicola	CBS 502.90^{T} of IMI 273194	*Oryza sativa*	Colombia	HF934949	LT715878	Restrepo et al. 2018
	CBS 376.76	*Oryza sativa*	Turkey	LT837456	LT883535	Restrepo et al. 2018
	BRIP 16229; CBS 132709^{IT}, IMI 354683^{T} of *E. fusiforme*	*Echinochloa crus-galli*	Australia	LT837455	LT715877	Restrepo et al. 2018
E. pedicellatum	CBS 322.64; MUCL 9617^{T}	*Triticum aestivum*	USA	KT265258	LT715902	Restrepo et al. 2018
	CBS 375.76	*Oryza sativa*	Turkey	KT265259	LT715879	Restrepo et al. 2018
E. protrudens	BRIP 14814; CBS 132710^{IT}, IMI 316693^{T}	*Dactyloctenium aegyptium*	Australia	LT631308	LT715880	Restrepo et al. 2018

	BRIP 14816	*Dactyloctenium aegyptium*	Australia	LT631309	LT715881	Restrepo et al. 2018
E. rostratum	BRIP 16078	*Spinifex hirsutus*	Australia	LT837826	LT882563	Restrepo et al. 2018
	BRIP 29236c	*Hordeum vulgare*	Australia	LT837492	LT882577	Restrepo et al. 2018
	CBS 188.68	unknown	South Africa	LT837839	LT882549	Restrepo et al. 2018
	CBS 273.52; IMI 048842; MUCL 18221; MUCL 9619	*Pennisetum spicatum*	Zambia	LT837830	LT882558	Restrepo et al. 2018
	CBS 297.80	*Sorghum bicolor*	Sudan	KT265244	LT715895	Restrepo et al. 2018
	CBS 323.64	*Zea mays*	USA	LT837833	LT715901	Restrepo et al. 2018
	CBS 325.87; ATCC 60408; CDC B-4030; NCMH 2445^{T} of *E. macginnisii*	*Homo sapiens*	USA	KT265237	LT715898	Restrepo et al. 2018
	CBS 571.73; ATCC 24775; IMI 175436^{A} of *S. prolata*	*Zea mays*	USA	LT837831	LT715892	Restrepo et al. 2018
	CBS 572.73; ATCC 24774; IMI 175435^{A} of *S. prolata*	*Zea mays*	Guatemala	LT837832	LT715893	Restrepo et al. 2018
	CBS 504.90; IMI 276558	*Sorghum bicolor*	Sudan	KT265243	LT883536	Restrepo et al. 2018
	CBS 128061 BRIP 12218, Lutt. 8868	*Zea mays*	USA	KT265240	LT715900	Restrepo et al. 2018
	LIPP EX1	*Lolium multiflorum*	Brazil	**To be assigned**		This study
	CBS 128063 BRIP 12223, SrA10	Ascospore isolate from Hay 3 × IMI 76563	USA	KT265239	LT883540	Restrepo et al. 2018
E. turcicum	CBS 387.58; NRRL 5240	*Zea mays*	USA	LT837483	LT883554	Restrepo et al. 2018

CBS 690.71; ET	*Zea mays*	Germany	LT837487	LT882581	Restrepo et al. 2018	

[1]ATCC: American Type Culture Collection, Bethesda, Maryland, USA; BRIP: Queensland Plant Pathology Herbarium, Brisbane, Australia; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; IMI: Kew Royal Botanical Gardens, Kew, England; MUCL: Mycothèque de L`Université Catholique de Louvain, Louvain-la-Neuve, Belgium; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil. ET: ex-epitype; IT: ex-isotype; IST: ex-isosyntype; NT: ex-neotype; PT: ex-paratype; ST: ex-syntype; T: ex-holotype; A: Authentic strain.

[2]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *gapdh*: partial glyceraldehyde-3-phosphate dehydrogenase.

Table 7. Details of *Pyrenophora* strains subjected to multi-gene DNA sequence analysis.

				GenBank accession numbers[2]			
Fungal Species	Strain no. [1]	Host	Location	ITS	LSU	*Gapdh*	References
Bipolaris yamadae	CBS 202.29	*Panicum miliaceum*	Japan	KM034830	MH866508	MH855044	Vu et al. 2019, Manamgoda et al. 2014
Pyrenophora avenicola	CBS 307.84^{T}	*Avena sativa*	Sweden	MK539972	MK540042	MK540180	Marin-Felix et al. 2019
P. biseptata	CBS 307.69	*Lolium multiflorum*	Germany	MK539973	MK540043	MK540181	Marin-Felix et al. 2019
	CBS 319.69	*Lolium perenne*	Germany	MK539974	MK540044	MK540182	Marin-Felix et al. 2019
P. bromi	CBS 311.68	*Bromus inermis*	Germany	MK539976	MH870851	MK540184	Vu et al. (2019), Marin-Felix et al. 2019
	DAOMC 127414	unknown	unknown	JN943666	JN940074	AY004839	Zhang & Berbee (2001), Hambleton (unpubl.data)
P. chaetomioides	CBS 279.31^{A}	unknown	unknown	MK539977	MK540045	MK540185	Marin-Felix et al. 2019
	CBS 195.31	unknown	unknown	MK539978	MH866633	MK540186	Vu et al. (2019), Marin-Felix et al. 2019
P. cynosuri	CBS 127918^{T}	*Cynosurus cristatus*	New Zealand	MK539980	MK540047	MK540188	Marin-Felix et al. 2019

P. dactylidis	DAOMC 92161	unknown	unknown	JN943667	JN940087	AY004812	Zhang & Berbee (2001), Hambleton (unpubl. data)
P. dictyoides	CBS 258.80	*Lolium perenne*	Germany	MK539981	MK540048	MK540189	Marin-Felix et al. 2019
	CBS 967.87	*Lolium perenne*	Germany	MK539982	MK540049	MK540190	Marin-Felix et al. 2019
P. erythrospila	CBS 312.69	*Lolium perenne*	Germany	MK539983	MK540051	MK540192	Marin-Felix et al. 2019
	CBS 108941	unknown	Canada	MK539984	MK540052	MK540193	Marin-Felix et al. 2019
P. fugax	CBS 509.77	unknown	Kuwait	MK539985	MK540053	MK540194	Marin-Felix et al. 2019
P. grahamii	CBS 128043	unknown	unknown	MK539987	MH876230	MK540196	Vu et al. (2019), Marin-Felix et al. 2019
	CBS 128044	unknown	unknown	MK539988	MH876231	MK540197	Vu et al. (2019), Marin-Felix et al. 2019
P. leucospermi	CBS 111083^{T}	*Leucospermum cordifolium*	South Africa	JN712467	JN712533	MK540198	Vu et al. (2019), Marin-Felix et al. 2019
	CBS 111505	*Leucospermum cordifolium*	South Africa	MK539989	JN712542	MK540199	Crous et al. (2011), Marin-Felix et al. 2019
P. lolii	CBS 240.48	*Lolium perenne*	Netherlands	MK539991	MK540055	MK540201	Marin-Felix et al. 2019
	CBS 128046	unknown	unknown	MK539993	MH876233	MK540203	Vu et al. (2019), Marin-Felix et al. 2019
P. nisikadoi	CBS 190.29^{ET}	*Briza minor*	Japan	KM257054	KM243296	KM257057	Manamgoda et al. (2014)
	CBS 119213	*Protea burchellii*	South Africa	EU552124	MK540056	MK540204	Marincowitz et al. (2008), Marin-Felix et al. 2019
P. nobleae	CBS 259.80	*Lolium multiflorum*	Germany	MK539994	MK540058	MK540206	Marin-Felix et al. 2019
	CBS 966.87	*Lolium multiflorum*	Germany	MK539995	MK540059	MK540207	Marin-Felix et al. 2019

	CBS 127936	unknown	unknown	MK539996	MK540060	MK540208	Marin-Felix et al. 2019
	LIPP DC1	*Lolium multiflorum*	Brazil	**To be assigned**			This study
P. novozelandica	CBS 127934^{T}	*Triticum* sp.	New Zealand	MK539997	MK540061	MK540209	Marin-Felix et al. 2019
P. phaeocomes	DAOMC 222769	unknown	unknown	JN943649	JN940093	–	Hambleton (unpubl. data), James et al. (unpubl. data)
P. poae	CBS 319.68^{A}	*Poa pratensis*	Germany	MK539998	MK540062	MK540210	Marin-Felix et al. 2019
	CBS 128045	*Poa pratensis*	USA	MK539999	MH876232	MK540211	Vu et al. (2019), Marin-Felix et al. 2019
P. pseudoerythrospila	CBS 127931^{T}	*Lolium* sp.	Germany	MK540000	MK540063	MK540212	Marin-Felix et al. 2019
P. semeniperda	BRIP 10941	*Triticum aestivum*	unknown	KJ415564	KJ415518	KJ415382	Tan et al. (2014)
	CBS 127927	unknown	unknown	MK540001	MK540064	MK540213	Marin-Felix et al. 2019
P. sieglingiae	CBS 127930	*Sieglingia decumbens*	Germany	MK540002	MK540065	MK540214	Marin-Felix et al. 2019
P. teres	CBS 228.76^{T} of *P. teres* f. *maculate*	*Hordeum vulgare*	Denmark	MK540003	MK540066	MK540215	Marin-Felix et al. 2019
	CBS 123929	*Hordeum* sp.	Hungary	MK540008	MK540070	MK540220	Marin-Felix et al. 2019
P. tetrarrhenae	CBS 127915	unknown	unknown	MK540010	MH877964	MK540222	Vu et al. (2019), Marin-Felix et al. 2019
	CBS 127924	unknown	unknown	MK540011	MH877965	MK540223	Vu et al. (2019), Marin-Felix et al. 2019
P. trichostoma	CBS 328.53	*Triticum* sp.	USA	MK540012	MK540072	MK540224	Marin-Felix et al. 2019
	CBS 391.54	unknown	unknown	MK540013	MK540073	MK540225	Marin-Felix et al. 2019

P. triseptata	CBS 128047	unknown	unknown	MK540015	MH877983	MK540227	Vu et al. (2019), Marin-Felix et al. 2019
	CBS 128048	unknown	unknown	MK540016	MH876234	MK540228	Vu et al. (2019), Marin-Felix et al. 2019
P. tritici-repentis	CBS 259.59^{SynT} of *P. tritici-vulgaris*	unknown	Japan	MK540017	MK540075	AM884276	Vu et al. (2019), Marin-Felix et al. 2019
	CBS 191.29	*Beckmannia* sp.	Japan	MK540018	MK540076	MK540229	Marin-Felix et al. 2019
P. variabilis	CBS 127920^{T}	*Poa trivialis*	Canada	MK540020	MK540078	MK540231	Marin-Felix et al. 2019
P. wirreganensis	CBS 109896	*Hordeum* sp.	Australia	MK540021	MK540079	MK540232	Marin-Felix et al. 2019

[1]CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; BRIP: Queensland Plant Pathology Herbarium, Brisbane, Australia; DAOMC: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil. A, ET, SynT and T indicates authentic, ex-epitype, ex-syntype and ex-type strains, respectively.

[2]ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: 28S nrRNA gene; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase.

Table 8. Details of *Pyricularia* strains subjected to multi-gene DNA sequence analysis.

Species	Strain no.[1]	Host	Location	GenBank Accession[2]			Reference
				ITS	*act*	*cmdA*	
Barretomyces calathae	CBS 129274= CPC 18464	*Catathea longifolia*	Brazil: Minas Gerais	KM484831	KM485162	KM485231	Klaubauf et al. 2014
Pyricularia ctenantheicola	GR0001 = Ct-4 = ATCC 200218	*Ctenanthe oppenheimiana*	Greece: Almyros, imported from Brazil via Netherlands	KM484878	KM485182	KM485252	Klaubauf et al. 2014

	GR0002[ET]	*Ctenanthe oppenheimiana*	Greece: Almyros, imported from Brazil via Netherlands	KM484879	KM485183	KM485253	Klaubauf et al. 2014
P. grisea	BR0029	*Digitaria sanguinalis*	Brazil: Goias, Goiana	KM484880	DQ240874	DQ240890	Klaubauf et al. 2014
	CBS 128304 = KACC 41641	*Echinochloa crus-galli var. Frumentacea*	Korea: Woanju	KM484881	KM485184	KM485255	Klaubauf et al. 2014
	CR0024	*Lolium perenne*	South Korea: Suwon	KM484882	KM485185	KM485256	Klaubauf et al. 2014
	JP0034 = NI980	*Digitaria smutsii*	Japan	KM484883	KM485186	KM485257	Klaubauf et al. 2014
	PH0055 = Dc88420	*Digitaria ciliaris*	Philippines: Sto Tomas, Batangas	KM484884	DQ240877	DQ240893	Klaubauf et al. 2014
	US0043 = G184	*Digitaria* sp.	USA: Delaware	KM484885	KM485187	KM485258	Klaubauf et al. 2014
P. oryzae	BF0028	*Paspalum* sp.	Burkina Faso	KM484886	KM485188	KM485259	Klaubauf et al. 2014
	BR0032	*Triticum* sp.	Brazil	KM484887	DQ240884	DQ240900	Klaubauf et al. 2014
	CBS 657.66	*Oryza sativa*	Egypt	KM484893	KM485194	KM485265	Klaubauf et al. 2014
	CD0067	*Leersia hexandra*	Cote d'Ivoire: Bouake	KM484896	KM485197	KM485268	Klaubauf et al. 2014
	CD0156	*Eleusine indica*	Cote d'Ivoire: Ferkessedougou	KM484897	KM485198	KM485269	Klaubauf et al. 2014

	CR0020	*Phleum pratense*	South Korea: Suwon	KM484898	KM485199	KM485270	Klaubauf et al. 2014
	CR0021	*Panicum miliaceum*	South Korea: Yongin	KM484899	KM485200	KM485271	Klaubauf et al. 2014
	GN0001	*Zea mays*	Gabon: Wey	KM484903	DQ240882	DQ240898	Klaubauf et al. 2014
	JP0028 = K76-79	*Eragrostis curvula*	Japan	KM484906	AF395961	KM485275	Klaubauf et al. 2014
	PH0014 = PO6-6	*Oryza sativa*	Philippines	KM484911	DQ240888	DQ240904	Klaubauf et al. 2014
	PH0035 = Bm8309 = PH0075	*Brachiaria mutica*	Philippines: Los Banos	KM484912	KM485207	KM485280	Klaubauf et al. 2014
	PH0079 = GPr8212	*Panicum repens*	Philippines	KM484919	KM485214	KM485287	Klaubauf et al. 2014
	LIPP PY2	*Lolium multiflorum*	Brazil		**To be assigned**		This study
	RW0012	*Eleusine coracana*	Rwanda: Kunynya	KM484922	AF395959	AF396014	Klaubauf et al. 2014
P. penniseticola	BF0017	*Pennisetum typhoides*	Burkina Faso: Kamboinse	KM484925	DQ240878	DQ240894	Klaubauf et al. 2014
	CD0086	*Pennisetum typhoides*	Cote d'Ivoire: Bouake	KM484926	DQ240879	DQ240895	Klaubauf et al. 2014
	CD0143	*Digitaria exilis*	Cote d'Ivoire: Odienne	KM484927	KM485219	-	Klaubauf et al. 2014
	CD0180	*Pennisetum* sp.	Cote d'Ivoire: Madiani	KM484928	DQ240880	DQ240896	Klaubauf et al. 2014

	ML0031[ET]	*Pennisetum typhoides*	Mali: Longorola Sikasso	KM484929	KM485220	-	Klaubauf et al. 2014
	ML0048	*Digitaria exilis*	Mali	KM484930	KM485221	-	Klaubauf et al. 2014
Pyricularia sp.	CBS 133598 = MAFF 305509 = NI919 (Leo-1J) = JP0036	*Leersia oryzoides*	Japan: Chiba	KM484940	AB274440	AB274473	Klaubauf et al. 2014
P. zingibericola	RN0001	*Zingiber officinale*	Reunion	KM484941	KM485229	KM485297	Klaubauf et al. 2014

[1]ATCC: American Type Culture Collection, Virginia, U.S.A.; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; MAFF: Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil. ET: ex-epitype.

[2]ITS: internal transcribed spacers and intervening 5.8S nrDNA; *act*: actin; *cmdA*: calmoldulin.

Table 9. Substitution models applied to the different phylogenetic analyses performed in this study.

	Loci						
Fungal genus	ITS	LSU	*tef1*	*gapdh*	*act*	*cmdA*	*his3*
Bipolaris	HKY+I+G		GTR+I+G	GTR+G			
Cercospora	K80+I				HKY+G	GTR+I	HKY+G
Colletotrichum	GTR+I+G				HKY		HKY+I
Curvularia	GTR+I+G		GTR+I+G	GTR+G			
Drechslera	SYM+I+G	GTR+I+G		HKY+I+G			
Exerohilum	HKY+I+G			GTR+I+G			
Pyricularia	GTR+G				SYM+G	HKY+G	

Substitution models used in the studies. GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; K80: Kimura 2-parameter; SYM: symmetrical model; Non-uniformity of evolutionary rates among sites were modeled by using a discrete Gamma distribution (+G) alone and with five rate categories and by assuming that a certain fraction of sites is evolutionarily invariable (+I).

Table 10. Total taxa (including the outgroup), unique site patterns in each locus, total unique site patterns, total trees generated and sampled (considering, a burn-in fraction of 0.25), for each phylogenetic study.

		Unique site patterns[1]							Total		
Fungal genus	Total taxa	ITS	*tef1*	*gapdh*	LSU	*act*	*cmdA*	*his3*	site patterns	Generated trees	Sampled trees
Bipolaris	57	49	55	87	—	—	—	—	191	7328	1832
Cercospora	51	1	—	—	—	21	32	29	83	5136	1284
Colletotrichum	27	17	—	—	—	30	—	59	106	3900	975
Curvularia	63	62	61	133	—	—	—	—	256	6904	1726
Exserohilum	32	82	—	99	—	—	—	—	181	836	209
Pyrenophora	47	208	—	218	23	—	—	—	449	6904	1726
Pyricularia	31	47	—	—	—	41	115	—	203	5832	1458

[1]ITS: internal transcribed spacers intervening 5.8S nrDNA; *tef1*: partial translation elongation factor 1-alpha gene; *gapdh*: partial glyceraldehyde-3-phosphate dehydrogenase gene; LSU: partial large subunit (28S) nrRNA gene; *act*: partial actin gene; *cmdA*: partial calmodulin gene; *his3*: partial histone H3 gene.

FIGURES

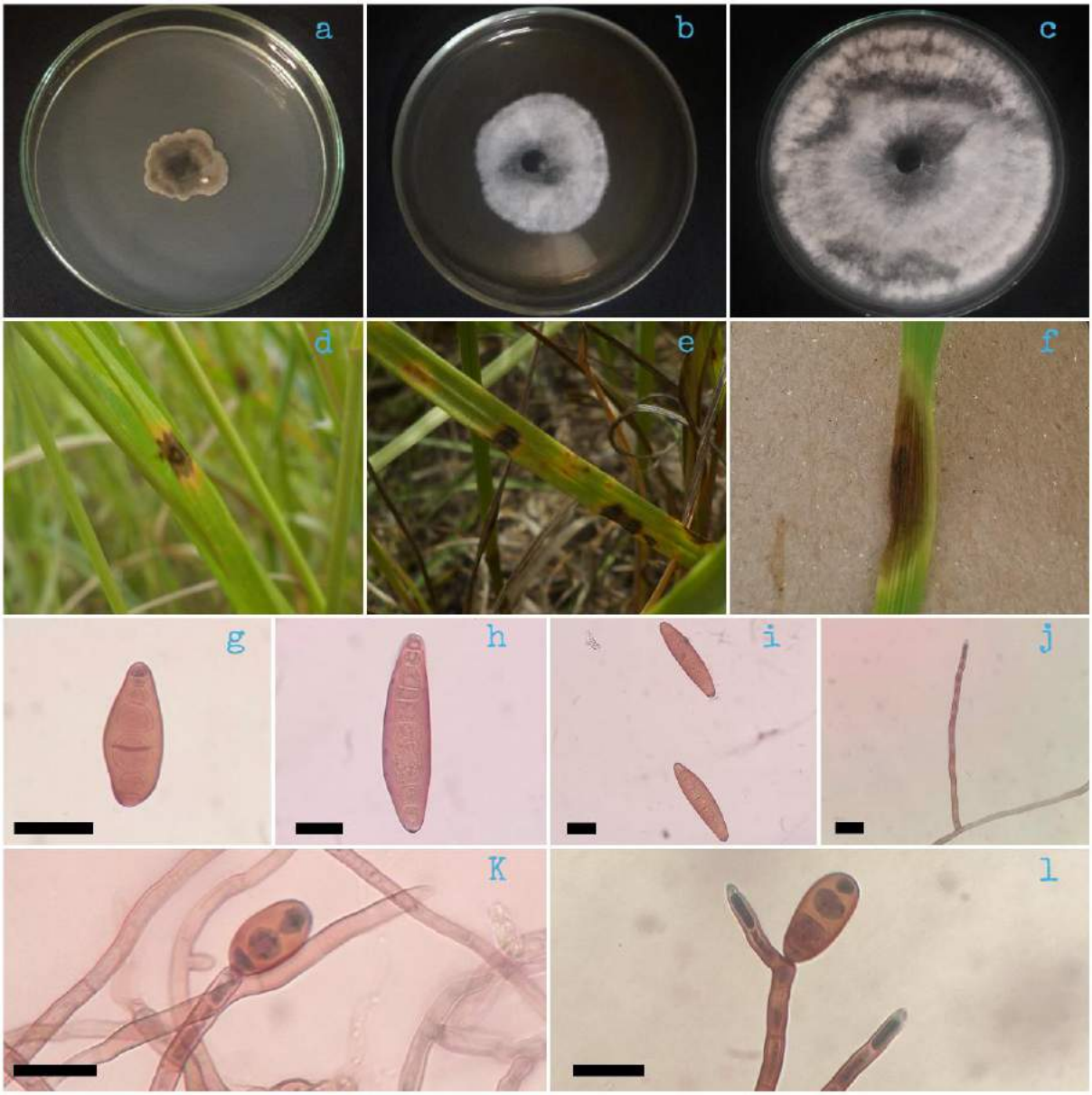

Fig. 1. *Bipolaris sorokiniana* (strain LIPP BI4); a. Culture on potato dextrose agar; b. Culture on malt extract agar; c. Culture on oatmeal agar; d-e. Brown leaf spot (field symptom); f. Brown leaf spot in the leaves of inoculated plants; g-i. Conidia; j. Conidiophore; k-l. Conidiophores and conidia; — Scale bars: g-l = 20 µm.

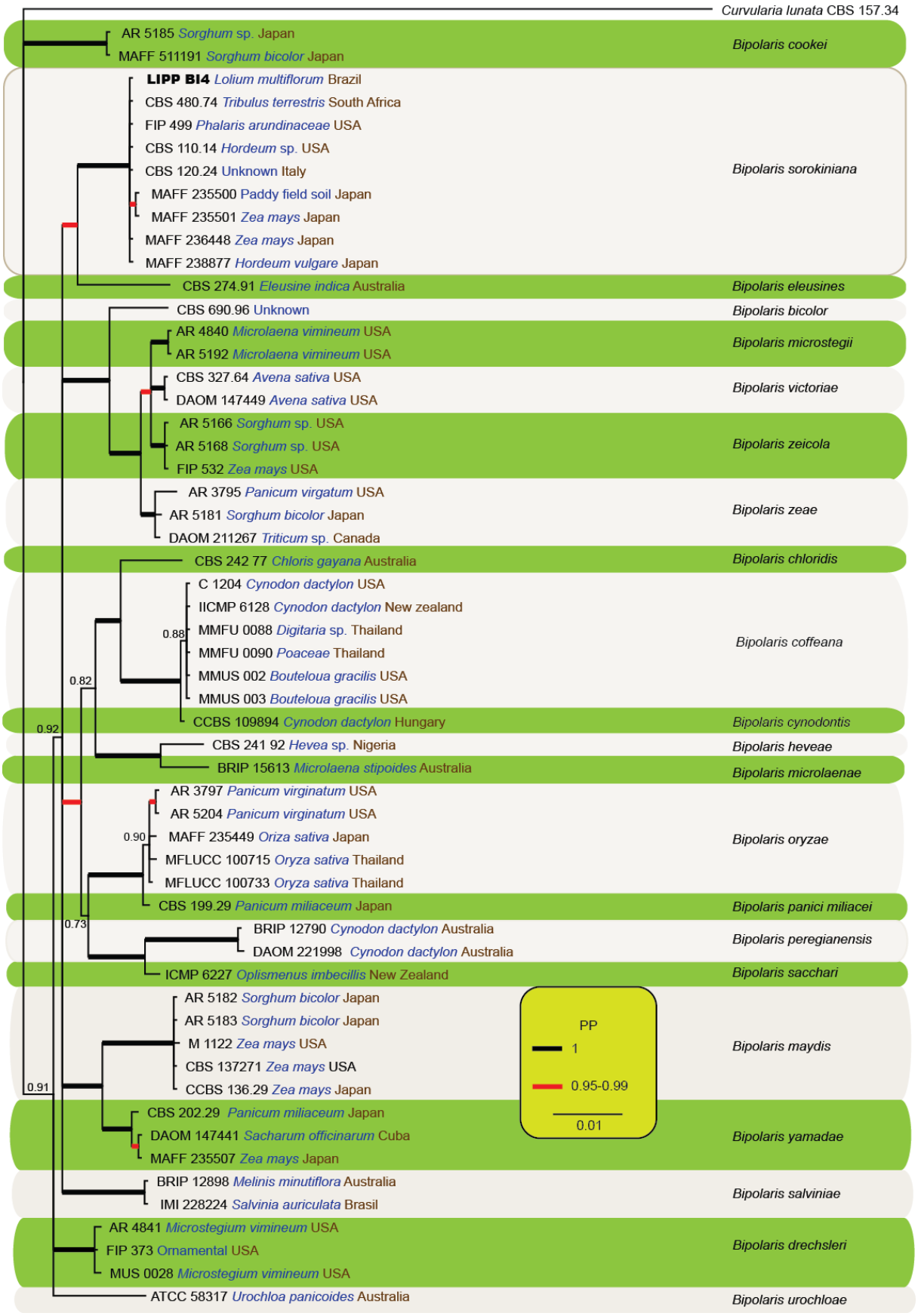

Fig. 2. Consensus phylogram (50 % majority rule) of *Bipolaris* species, from a Bayesian analysis of the combined 3-gene sequence alignment (ITS, *gapdh*, and *tef1*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar

indicates 0.01 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text and the country in brown text. The tree was rooted to *Curvularia lunata* (strain CBS 157.34).

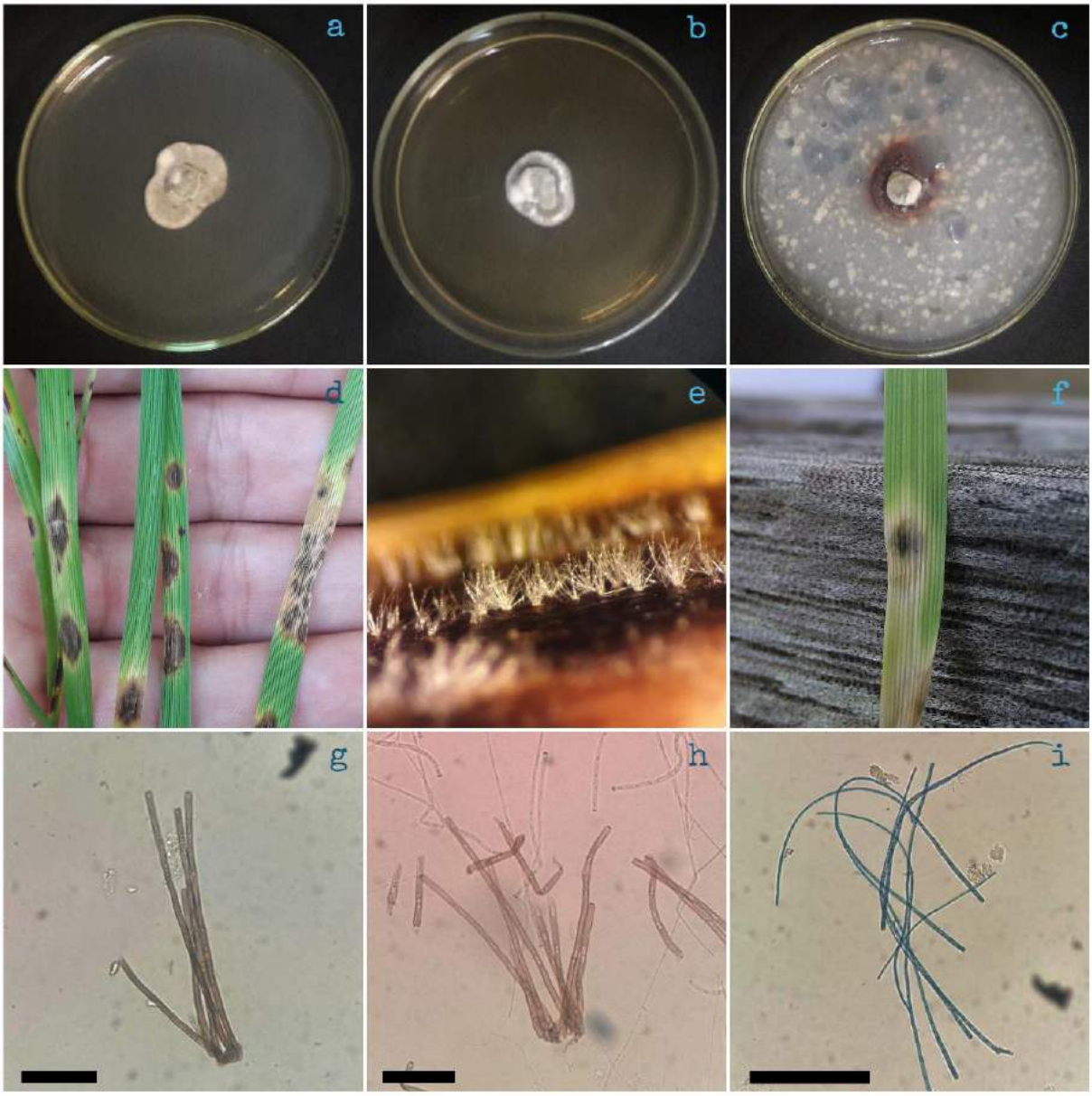

Fig. 3. *Cercospora* sp. nov. (LIPP C2A); a. Culture on potato dextrose agar; b. Culture on malt extract agar; c. Culture on oatmeal agar; d-e. Field symptoms showing parrot's eye leaf spot (PLS); f. Inoculated plants showing symptoms of PLS; g-h. Conidiophores; i. Conidia; — Scale bars: g-i = 50 µm.

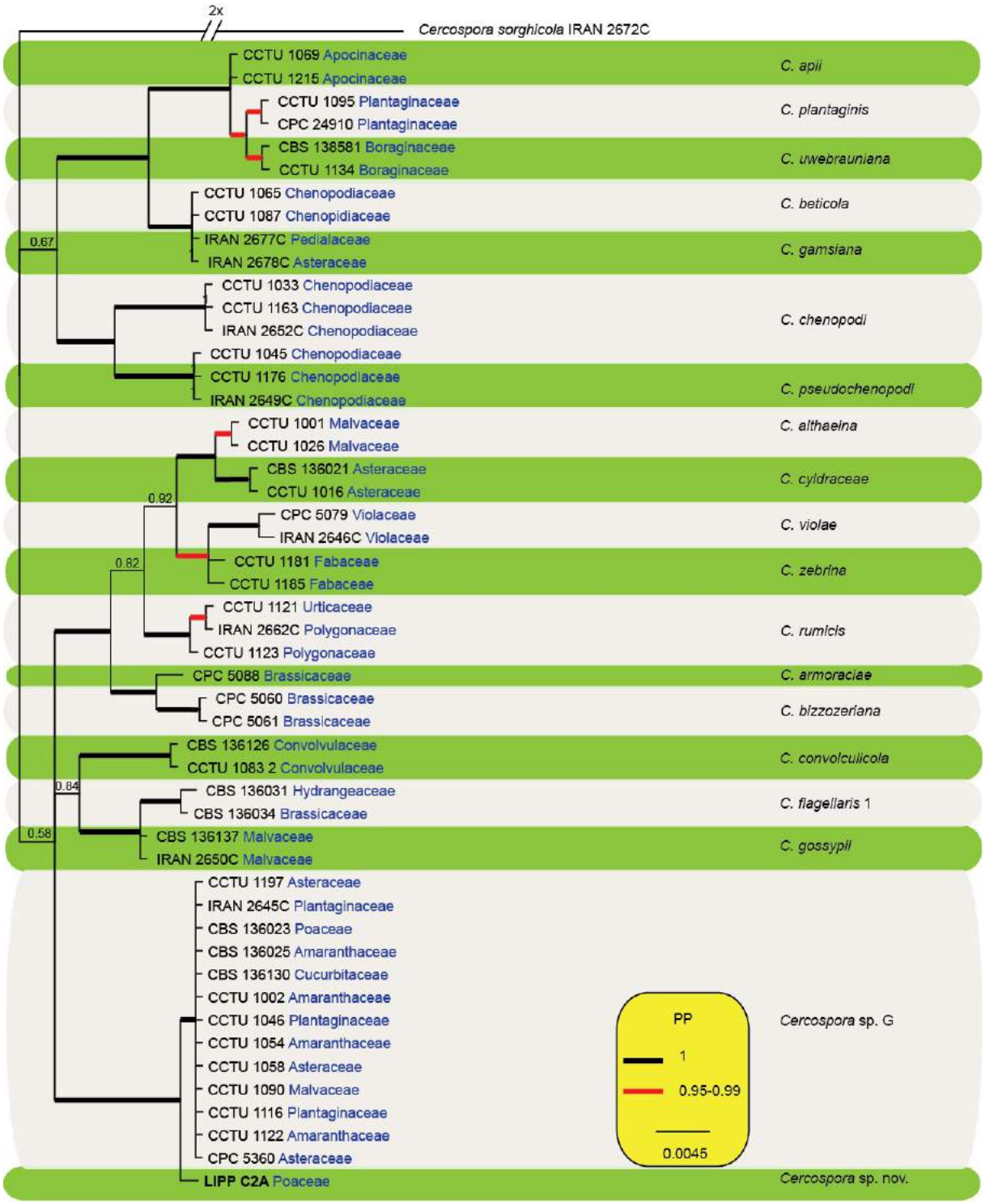

Fig. 4. Consensus phylogram (50 % majority rule) of *Cercospora* species, from a Bayesian analysis of the combined 4-gene sequence alignment (ITS, *act*, *cmdA* and, *his3*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.0045 expected changes per site. Isolate from Brazil is indicated in bold. Substrates family are indicated in blue text. The tree was rooted to *Cercospora sorghicola* (strain IRAN 2672C).

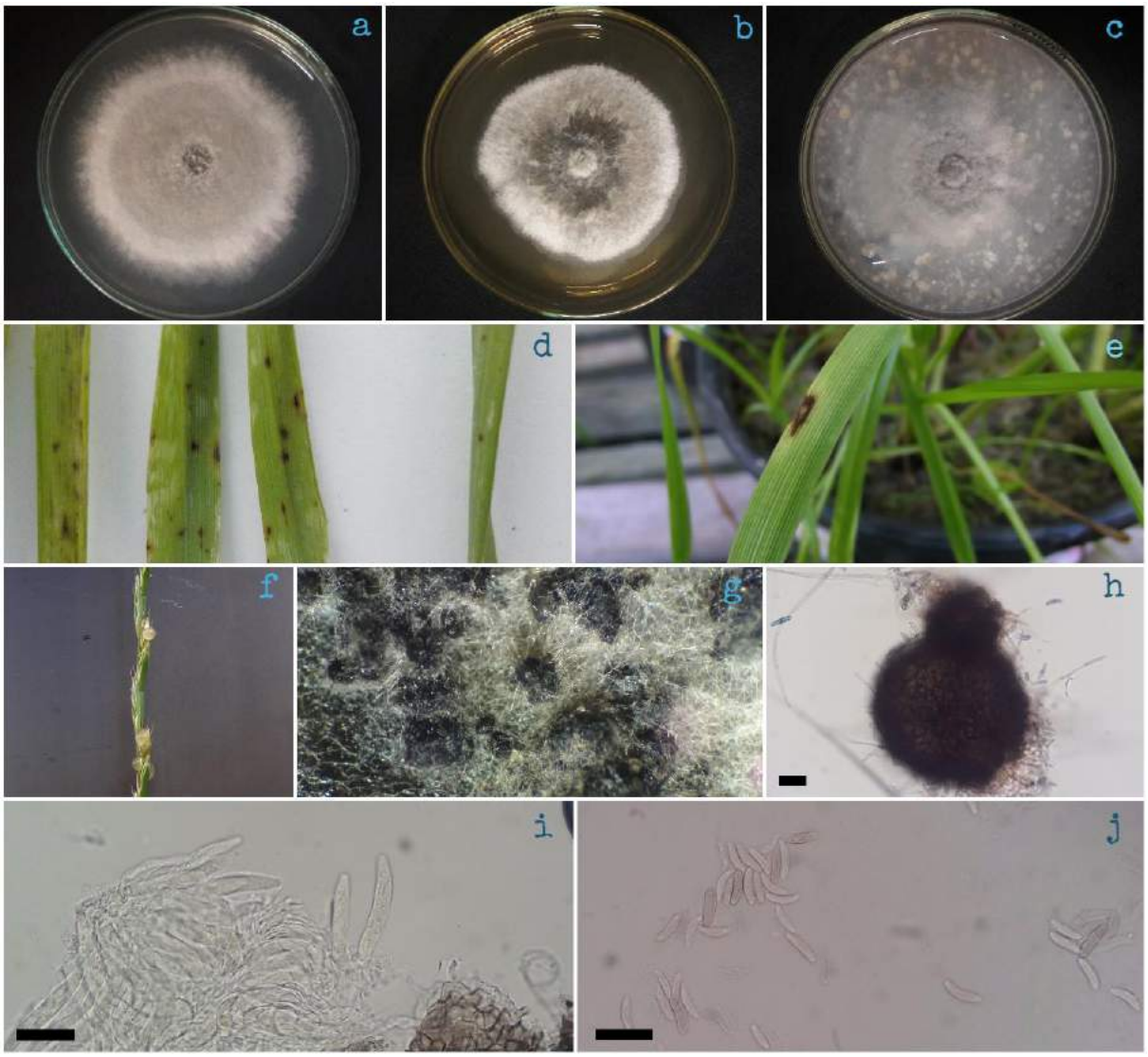

Fig. 5. *Colletotrichum plurivorum* (LIPP COLL1B); a. Culture on potato dextrose agar; b. Culture on malt extract agar; c. Culture on oatmeal agar; d. Field symptoms of *C. plurivorum*; e. Inoculated plants showing disease symptoms of *C. plurivorum* in the leaf; D. Inoculated plants showing disease symptoms in the spike; g-h, Ascomata; i. Asci; j. Ascospores; — Scale bars: h-j = 20 µm.

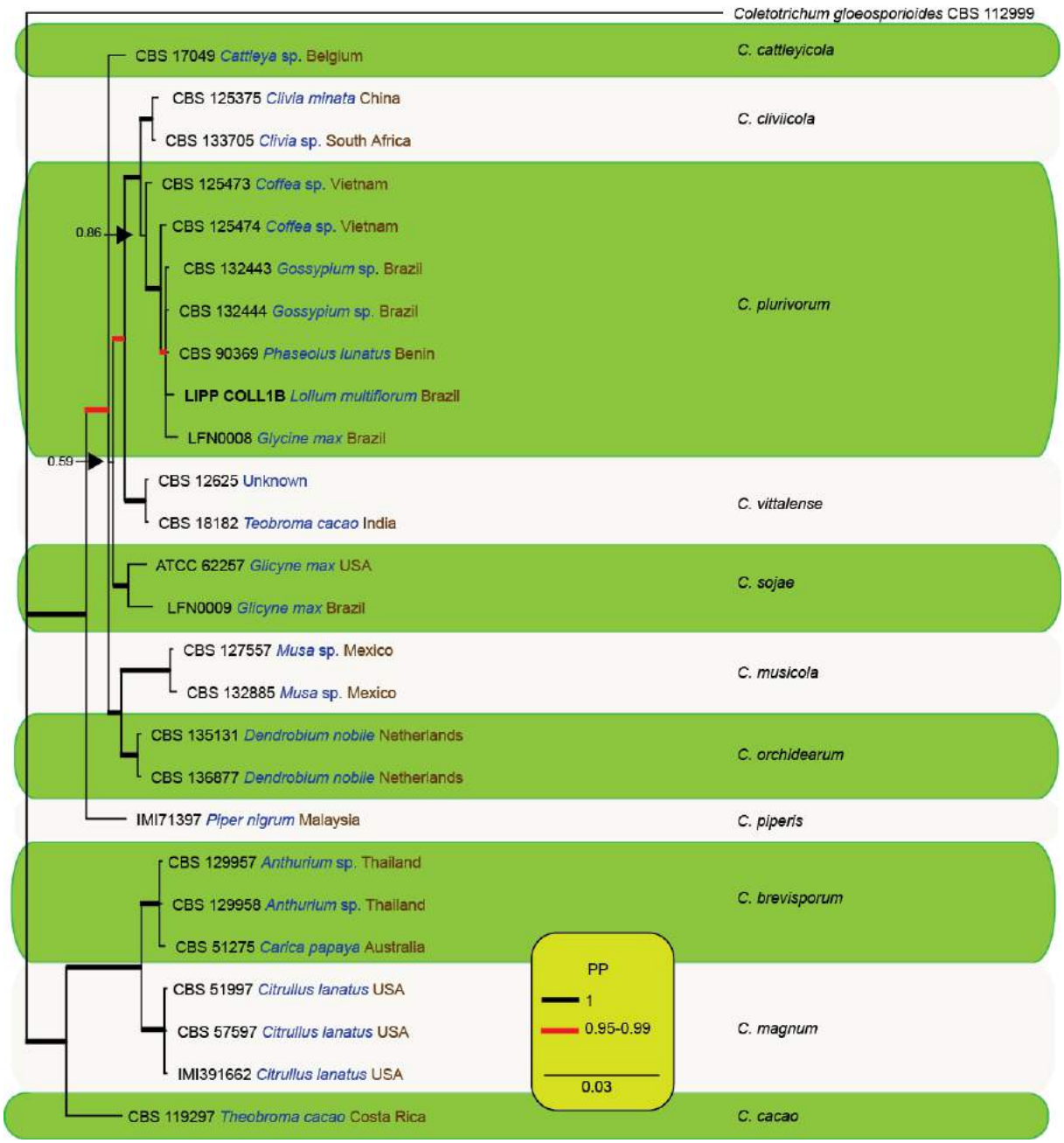

Fig. 6. Consensus phylogram (50 % majority rule) of *Colletotrichum* species, from a Bayesian analysis of the combined 3-gene sequence alignment (ITS, *his3*, and *act*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.03 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text and countries in brown text. The tree was rooted to *Coletotrichum gloeosporioides* (strain CBS 112999).

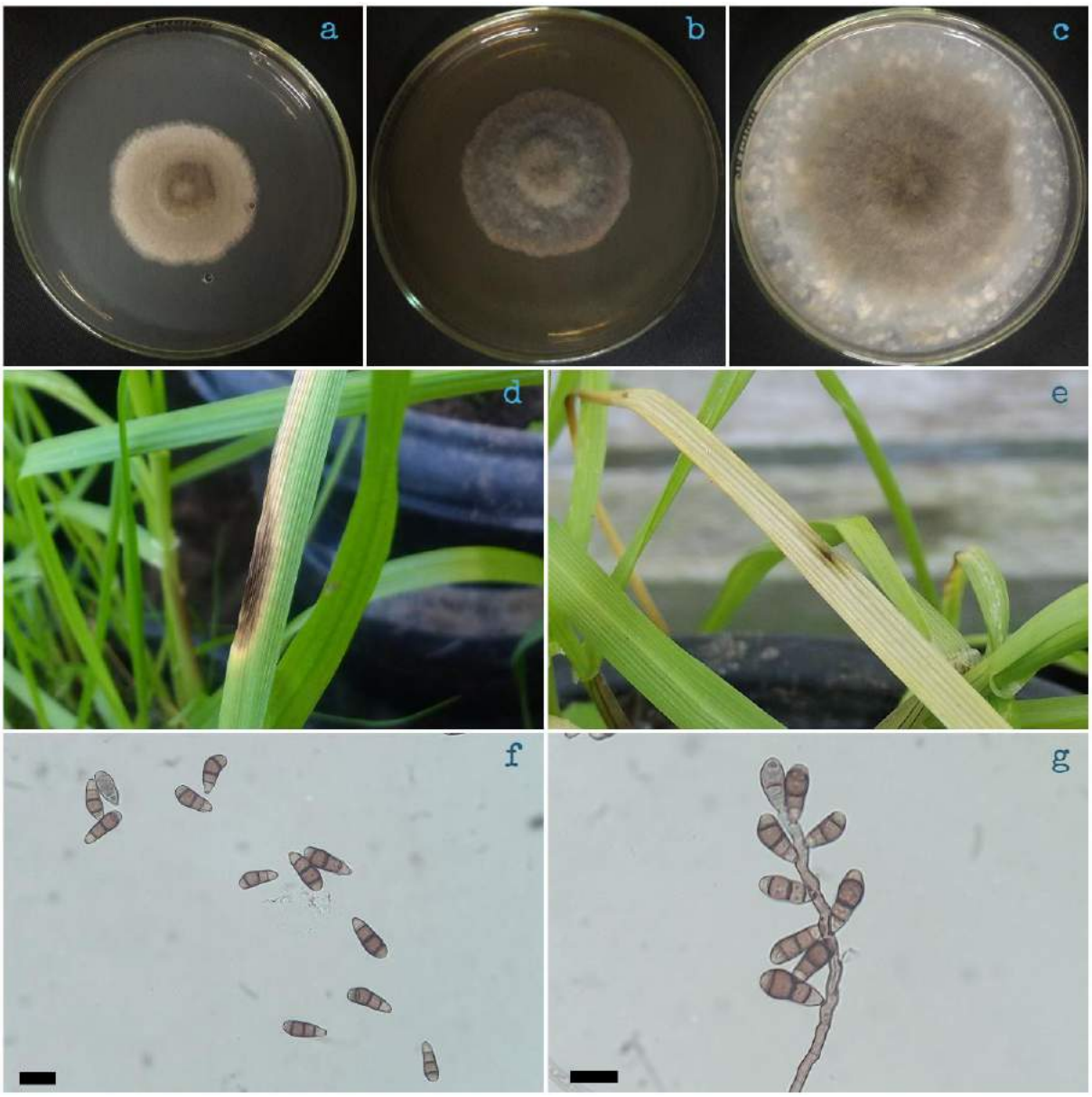

Fig. 7. *Curvularia* sp. nov. (LIPP CU1). a. Culture on potato dextrose agar: b. Culture on malt extract agar: c. Culture on oatmeal agar; d-e. inoculated plants showing disease symptoms on the leaf; f. Conidia; g. Conidiophore and conidias; — Scale bars: f-g = 20 µm.

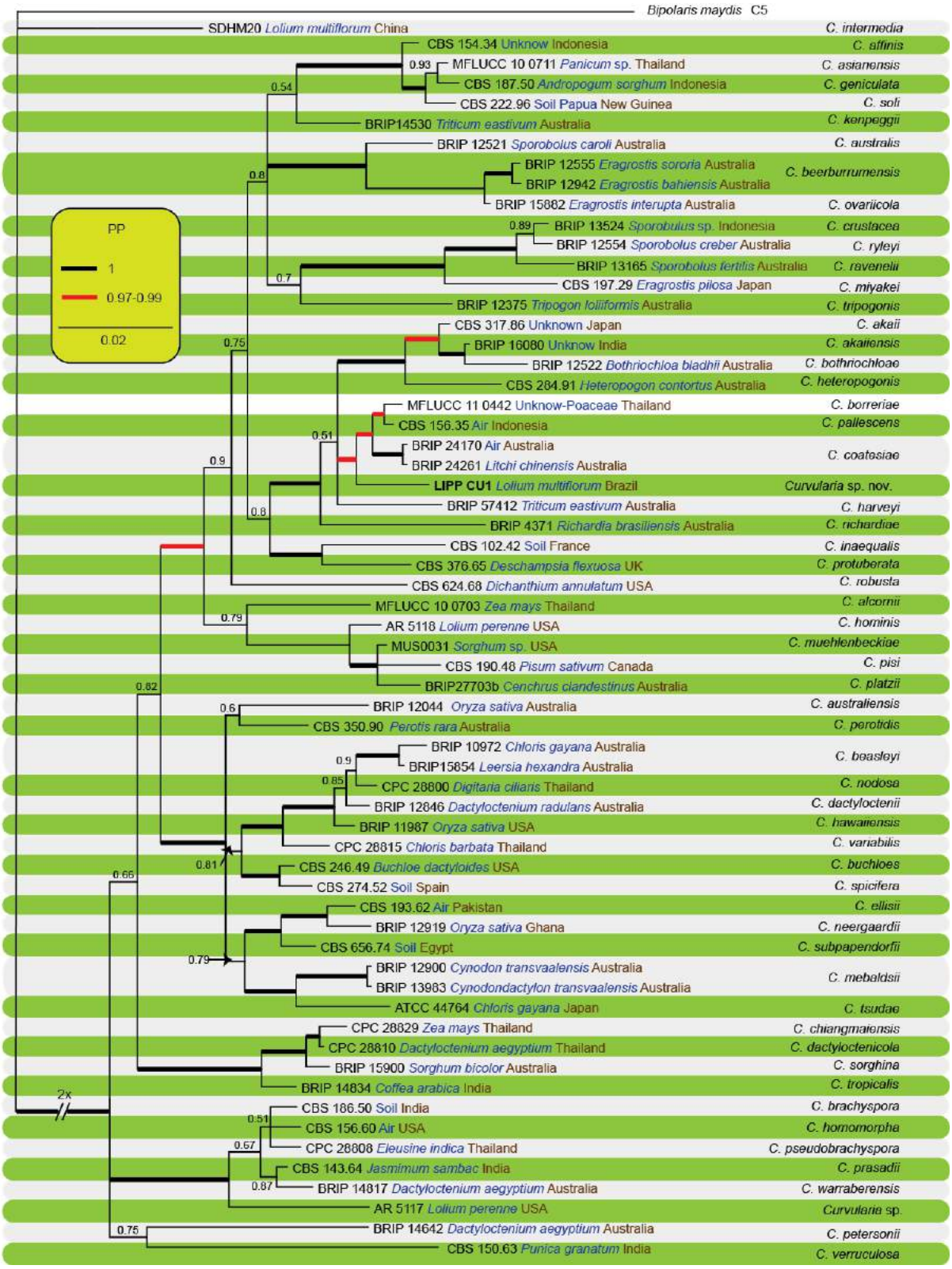

Fig. 8. Consensus phylogram (50 % majority rule) of *Curvularia* species, from a Bayesian analysis of the combined 3-gene sequence alignment (ITS, *gapdh* and, *tef1*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.02 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text and countries in brown text. The tree was rooted to *Bipolaris maydis* (strain C5).

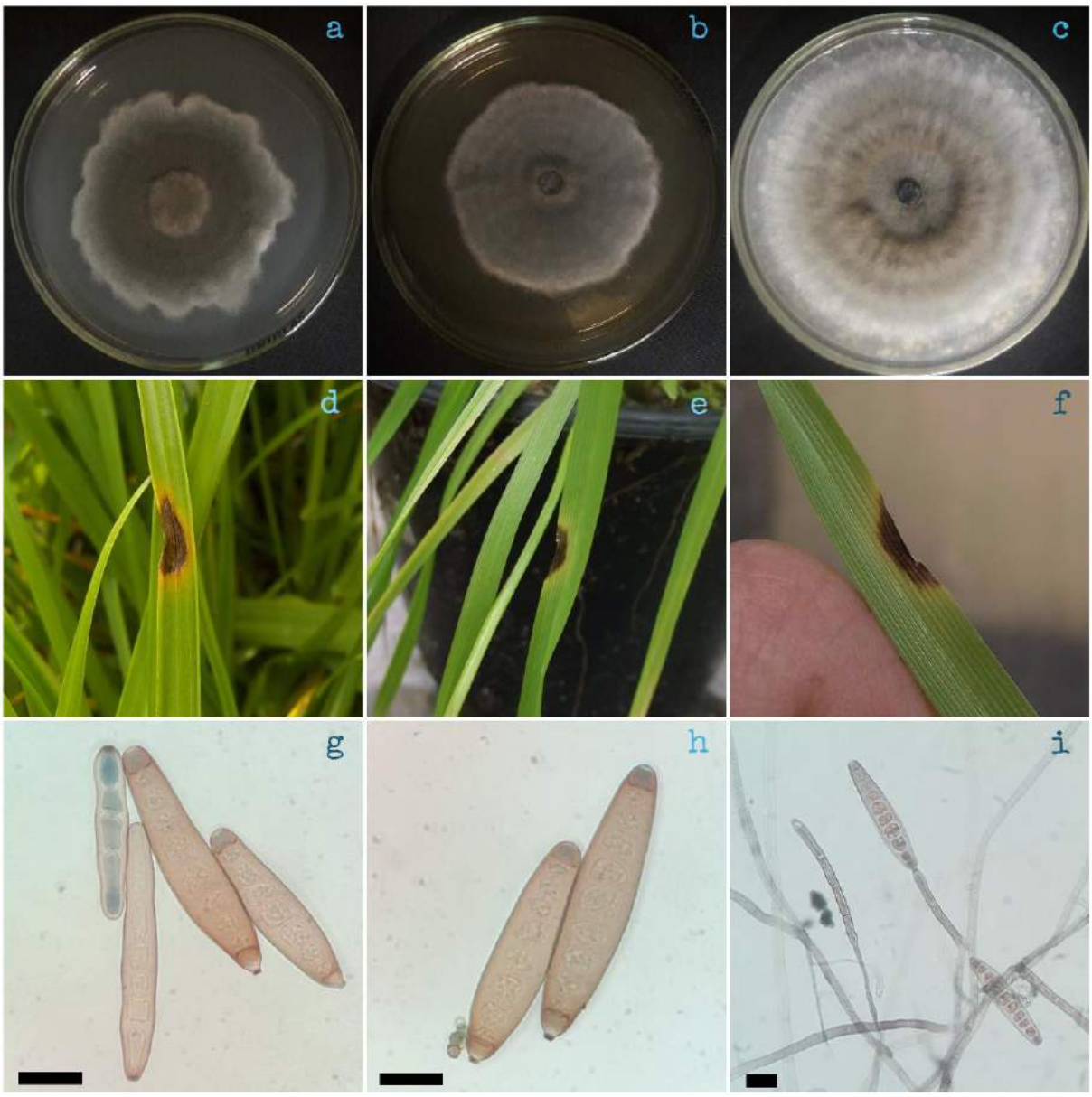

Fig. 9. *Exerohilum rostratum* (LIPP EX1). a. Culture on potato dextrose agar; b. Culture on malt extract agar: c. Culture on oatmeal agar; d. Disease symptoms of Exserohilum leaf spot (ELS) under field conditions: e. Inoculated plants showing symptoms of ELS; g-h. Conidia; i. Conidiophores and conidias; — Scale bars: g-i = 20 µm.

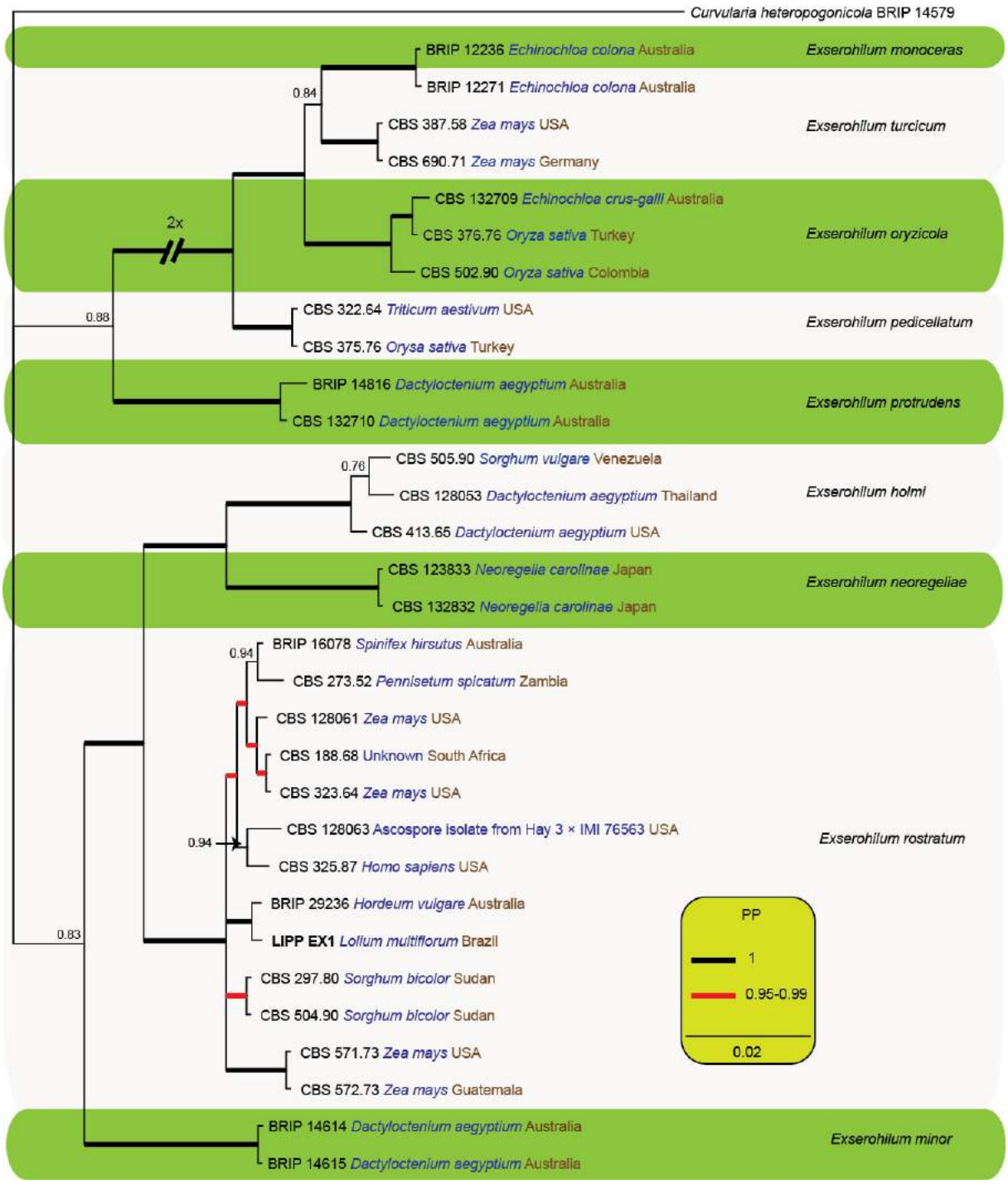

Fig. 10. Consensus phylogram (50 % majority rule) of *Exserohilum* species, from a Bayesian analysis of the combined 2-gene sequence alignment (ITS and *gapdh*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.02 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text and counties in brown text. The tree was rooted to *Curvularia heteropogonicola* (strain BRIP 14579).

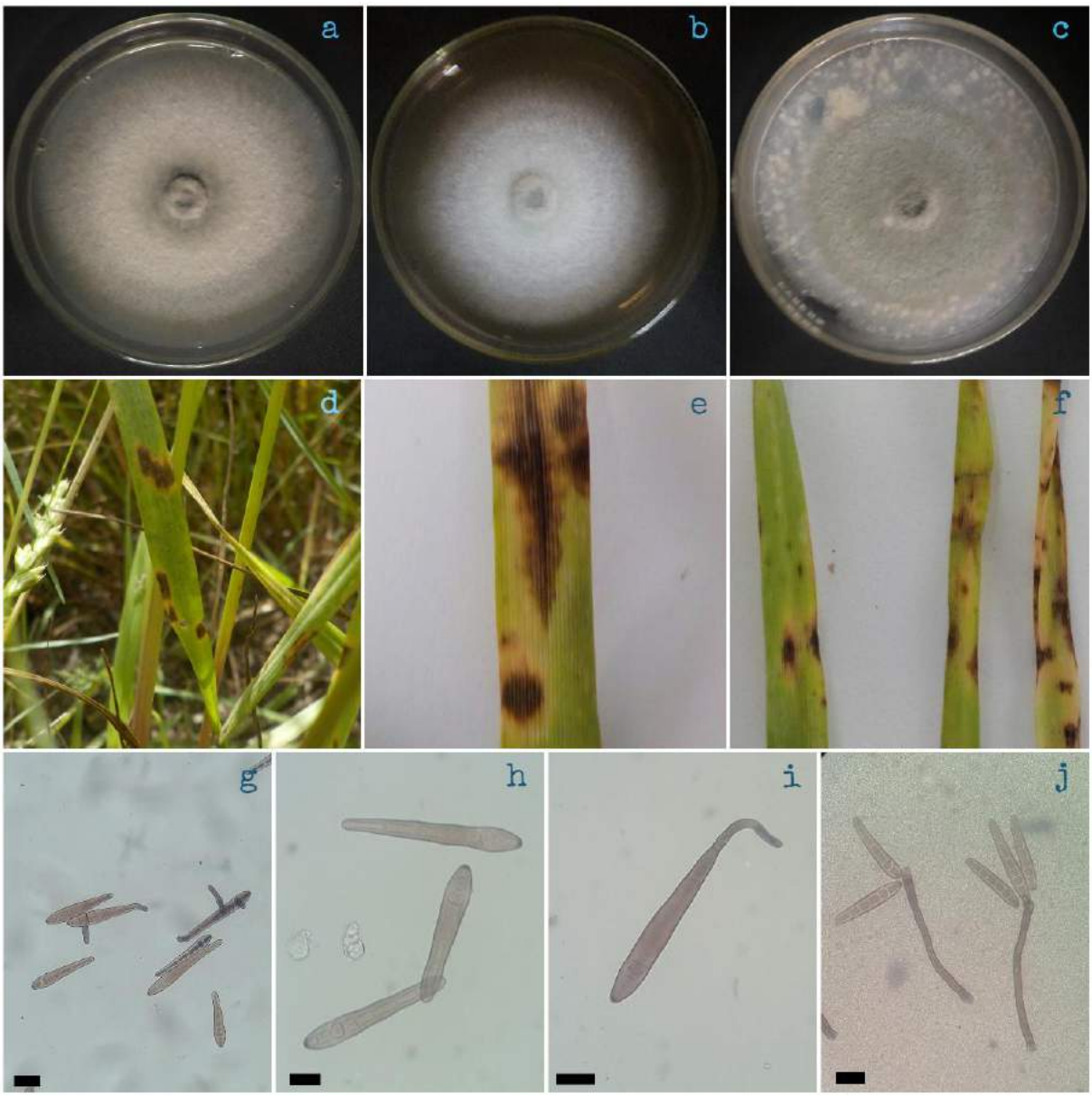

Fig. 11. *Pyrenophora nobleae* (LIPP DC1); Culture on potato dextrose agar; b. Culture on malt extract agar; c. Culture on oatmeal agar; d. Disease symptons under field conditions of yellow leaf spot (YLS); e-f. Inoculated plants showing symptoms of YLS; g-i. Conidia; j. Conidiophores and conidias; — Scale bars: g-j = 20 µm.

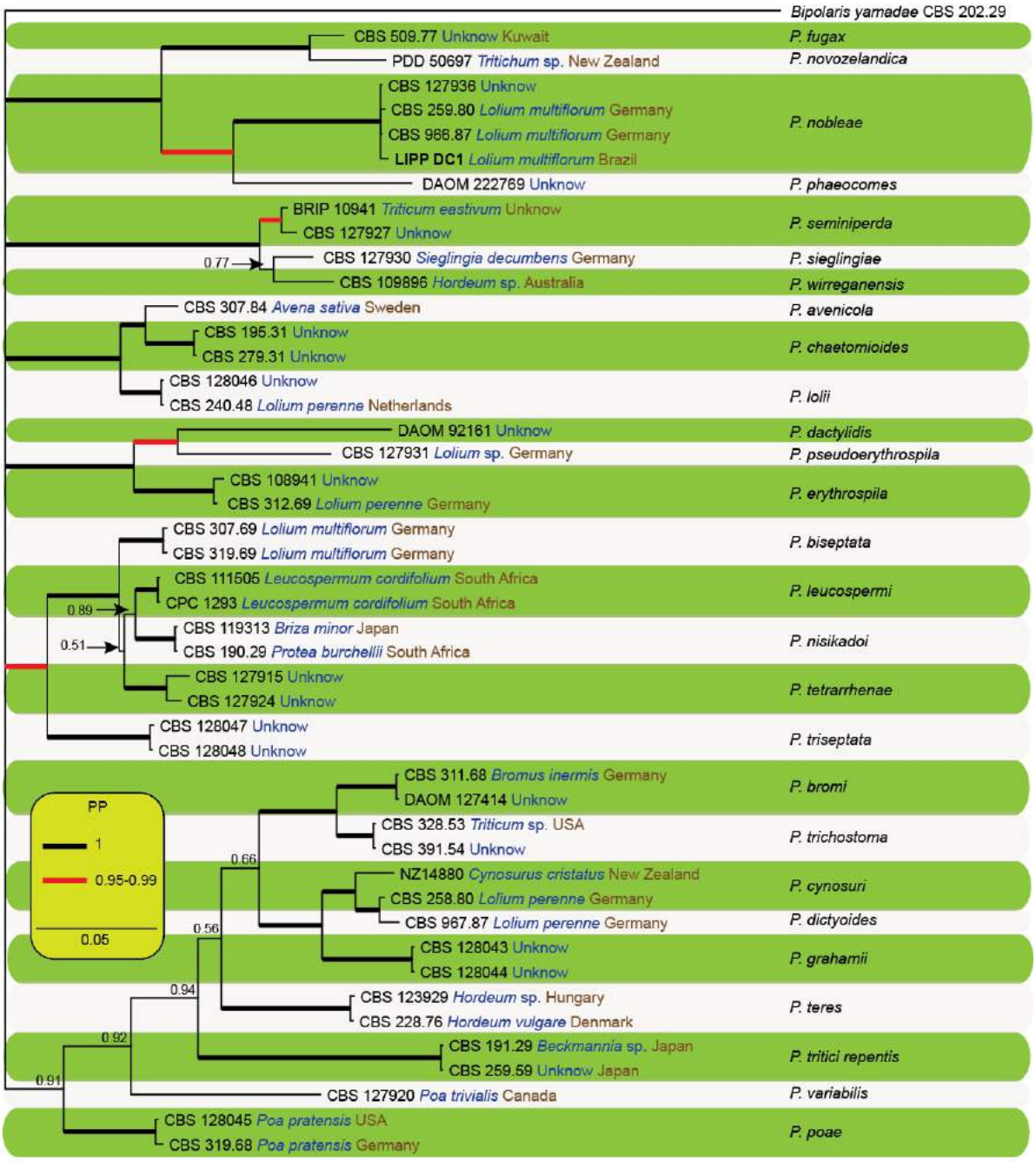

Fig. 12. Consensus phylogram (50 % majority rule) of *Pyrenophora* species, from a Bayesian analysis of the combined 3-gene sequence alignment (ITS, LSU, and *gapdh*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.05 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text and countries in brown text. The tree was rooted to *Bipolaris yamadae* (strain CBS 202.29).

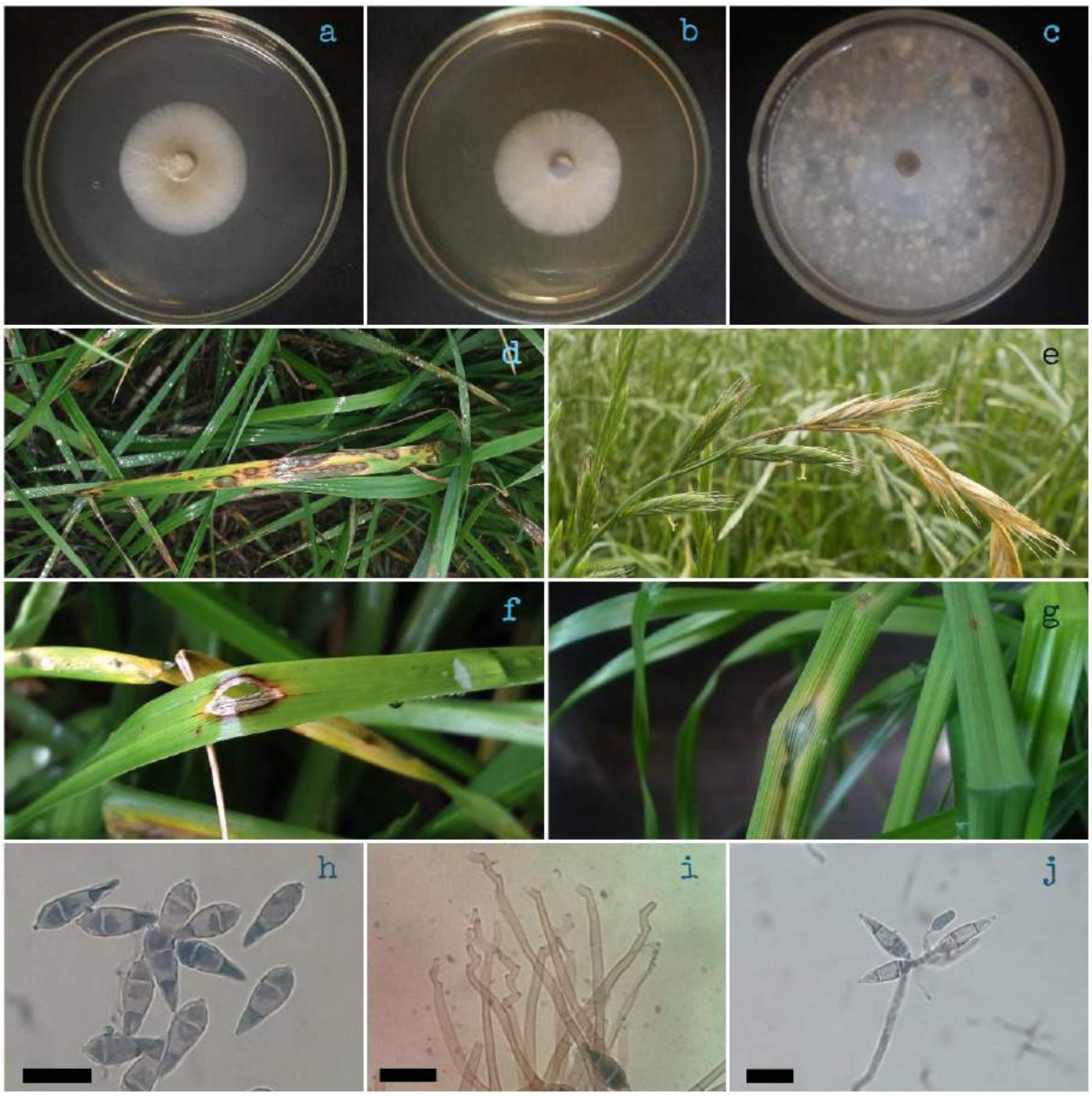

Fig. 13. *Pyricularia oryzae* (LIPP EX1); Culture on potato dextrose agar; b. Culture on malt extract agar; c. Culture on oatmeal agar; d-e. Field symptoms of gray leaf spot (GLS); f-g. Inoculated plants showing symptoms of GLS; h. Conidias; i. Conidiophores; j. Conidiophore and conidias; — Scale bars: h-j= 20 µm.

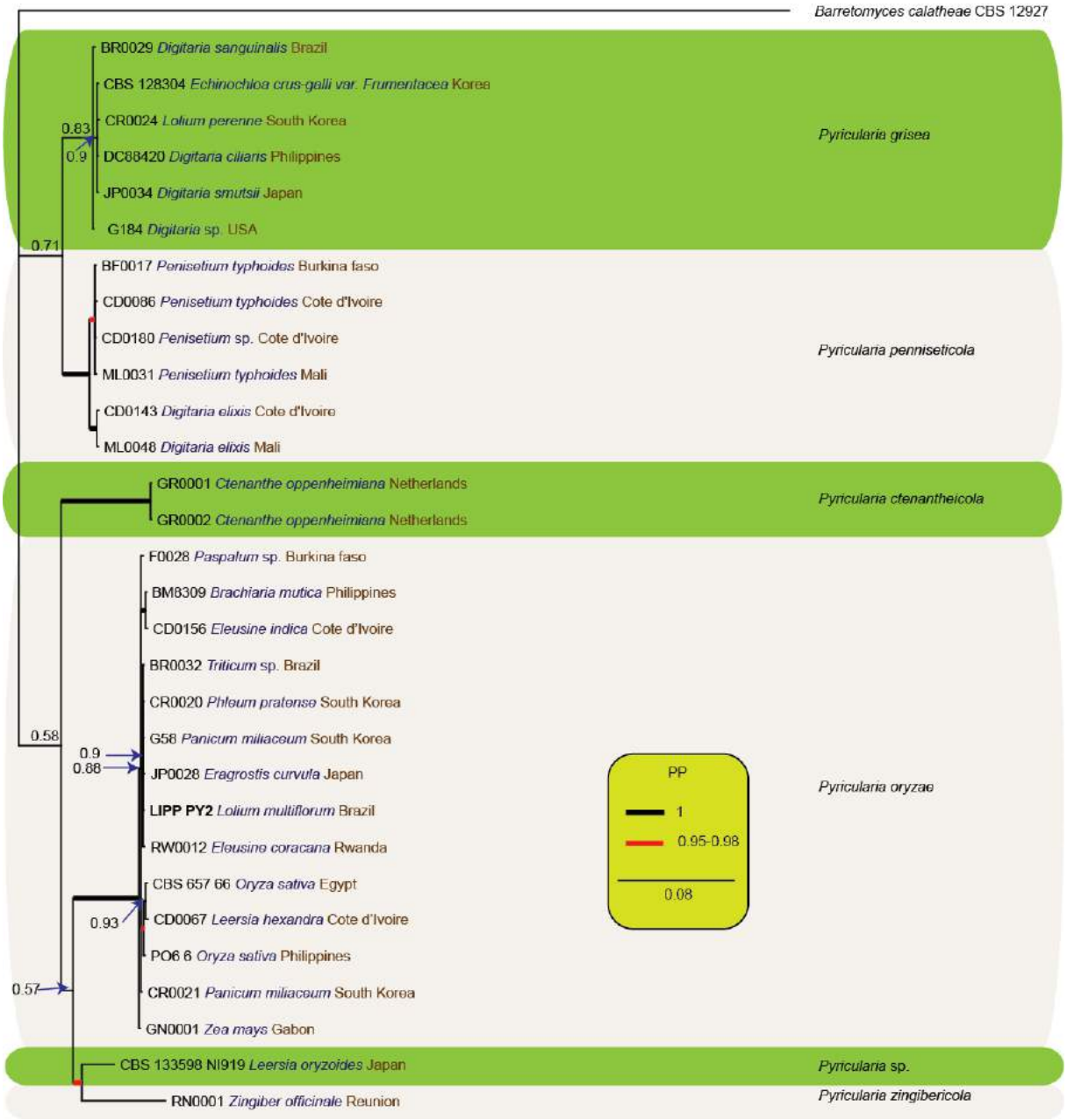

Fig. 14. Consensus phylogram (50 % majority rule) of *Pyricularia* species, from a Bayesian analysis of the combined 3-gene sequence alignment (ITS, *act* and *cmdA*). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.08 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text and countries in brown text. The tree was rooted to *Barretomyces calatheae* (strain CBS 129274).

This study was formatted under the guidelines of the Journal **Biological control**

Coated seeds with *Trichoderma atroviride* and soil amended with silicon improve the resistance of *Lolium multiflorum* against to *Pyricularia oryzae*

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ABSTRACT

Ryegrass (*Lolium multiflorum* L.) is a forage with a high content of protein, palatability, and digestibility. In winter, it is frequently used in southern Brazil as food for livestock. In this region of high humidity, the gray leaf spot, caused by *Pyricularia oryzae*, have been associated as the main biotic problem. In this study, we aimed to evaluate the effect of three types of management, namely, seed coating with *Trichoderma atroviride* (T), the application of silicon in the soil (Si), and the combination of both treatments (TSi) to reduce the severity of the gray leaf spot. In the first stage, the capacity of 10 native isolates of *Trichoderma* spp. were evaluated *in vitro* to directly inhibit *P. oryzae* from tests of dual cultures (DC), antibiosis for volatile (Vol), and non-volatile compounds (Nvol). *In vivo* tryals involved the ability to induce resistance responses, such as severity (SEV) and number of lesions (NL) per cm^2 of leaf area. In the second stage, the efficacy of T, Si, and TSi was tested under greenhouse conditions. The lesion expansion rate (LER), the relative efficiency of infection (RIE), SEV and NL were measured (after inoculation with *P. oryzae*), and in parallel was quantified (at 24, 48, 72, and 96 hours) the activities of the enzyme superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and phenylalanine ammonia-lyase (PAL). The strain TR10 (identified by polyphasic taxonomy as *T. atroviride*), showed one of the highest inhibition values in Vol (23%) and DC (35%). This strain (TR10) kept the lowest levels of SEV (5.5%) and NL (3) that contrasted with other strains according to the analysis of variance. Under greenhouse conditions, significant differences ($P < 0.0001$) were found with LER, RIE, NL and SEV in T, Si, and TSi concerning TE (control treatment). The defense response of T was related to the activities of CAT, POX, and PAL (mainly), while for Si others mechanism, that were not evaluated in this study, might contributed to disease reduction, and TSi did not show an additive effect. The

treatment of seeds with *T. atroviride* and the application of Si in the soil improve the resistance in *L. multiforum* against infection by *P. oryzae*.

Keywords**:** gray leaf spot, biological control, plant defense, induction of resistance, antimicrobial compounds.

1. Introduction

Annual pastures represent one of the most economically profitable and nutritious sources for livestock feeding during the winter period in southern Brazil (Hahn et al., 2015). The annual ryegrass (*Lolium multiflorum*) is one of the most cultivated pasture, due to its high palatability and digestibility (Xue et al., 2017). Additionally, ryegrass have natural reseeding, which reduces costs and facilitates its restoration (Trezzi et al., 2007).

The cultivar BRS Ponteio (*Lolium multiflorum*) has been one of the most used, due to the high yields in dry matter (Hahn et al., 2015), which is a desirable feature to ensure satisfactory levels of livestock production and keep costs low, and at the same time high-quality forage should be available throughout the year (Tambara et al., 2017). On the other hand, BRS Ponteio demonstrated susceptibility to gray leaf spot disease caused by different isolates of *Pyricularia* spp., and the losses derived from this problem can reach up to 100% in some regions (Donizeti et al., 2017). Moreover, there are no fungicides legally approved by the Brazilian Ministry of Agriculture against any pathogens in this forage grass, and pesticides are currently one of the biggest public health problems in Brazil due to the size of the population exposed in agricultural areas (Rigotto et al., 2014). In this sense, it is necessary to develop ecologically and sustainable alternative to guarantee forage production to increase production potential.

One of the widely studied alternatives is the biological control, which involves the use of beneficial microorganisms or biological control agents (BCA) to attack and control plant pathogens and the diseases they cause (Singh et al., 2014). *Trichoderma* spp. is the main genus applied to combat a wide range of plant diseases. Species in this genus acts by activating single or multiple control mechanisms, including mycoparasitism, enzyme production, competition (nutrients/space) and induced resistance in plants, that directly or indirectly interfere with the target pathogen (Nusaibah and Musa, 2019). Considering these advantages, the coating of seeds with BCA has emerged as a feasible way of administering the antagonist for the management of plant diseases (Mastouri et al., 2010). This practice forms a layer of germinated conidia on the seed surface that offers protection and also results in a rapid and uniform seedling emergence (Singh et al., 2014).

Silicon has been reported to improve the defense of plants against pathogens caused by fungi, bacteria, and viruses (Debona et al., 2017a; Luyckx et al., 2017). There are several ways in which silicon acts, but the most usual are physical-mechanical resistance, induction of biochemical and molecular responses (Debona et al., 2017b; Wang et al., 2017). Physical responses have been evidenced in the *Magnaphorte grisea -Oryza sativa* pathosystem, in which silicon works by generating lower penetration sites and reducing the relative efficiency of infection (Rodrigues et al., 2004; Seebold et al., 2001), and lower severity by accumulating mainly in the intercellular spaces, the middle lamella, and cells epidermal (Kim et al., 2002).

Biochemical responses induced in plants by silicon includes the increase in the activity of defense-related enzymes (polyphenol oxidase, chitinase, glucanase, peroxidase and phenylalanine ammonia-lyase); induction of production of antimicrobial compounds (phenolic compounds, flavonoids, phytoalexins, and pathogenesis-related proteins) and regulation of systemic signals, such as salicylic acid, jasmonic acid and ethylene (Debona et al., 2017b) Wang et al., 2017). These mechanisms have been evidenced in several families of plants including gramineous such as perennial ryegras (*Lolium perenne*), wheat (*Triticum eastivum*), and rice (*Oryza sativa*) in pathogens that cause leaf diseases such as *P. oryzae*, *Magnaphorte grisea*, *Pyrenophora tritici-repentis*, and *Bipolaris sorokiniana* (Cai et al., 2008; Domiciano et al., 2010, 2015; Dorneles et al., 2018; Rahman et al., 2015).

According to Rodrigues and Datnoff (2005), silicon sources and their management practices should be developed and practiced in integrated pest management programs for those crops in which silicon has been shown to have a positive effect. However, there are currently no studies demonstrating its effectiveness in *L. multiflorum* against *P. oryzae*. Recently, this pathogen was reported in Brazil in this forage grass. (see study I).

Under this scenario, this work aimed: 1) to find a native isolated of *Trichoderma* with high performance to control (directly/indirectly) *P. oryzae* and 2) to test the effectiveness of the application of silicon in the soil, alone and in combination with the pretreatment of seeds with *Trichoderma*, to reduce gray leaf spot disease, and 3) investigate possible related biochemical responses.

2. Materials and methods

2.1 Study area

The experiment was conducted in three places. Soil sampling for the isolation of the antagonists were carried out at the Agriculture Center of Palma (31°48'06.4"S 52°30'18.6"W), while the *in vitro* phase was developed in the Plant-Pathogen Interaction Laboratory and the *in vivo* phase, in the greenhouse of the Crop Protection Department. These facilities belonging to the Federal University of Pelotas.

2.2 First phase (in vitro)

This experimental phase included the isolation and preliminary identification of the *Trichoderma* strains. Besides, it also included the evaluation of growth, and its performance in confrontation tests (mycoparasitism) and indirect (antibiosis) on the target pathogen (*P. oryzae*), to know its potential as an antagonist.

2.2.1 Isolation and preliminary identification

The isolation of *Trichoderma* spp. was from rhizospheric soil of *Lolium multiflorum* plants (collected at the place described above), using the plate dilution method, as described by Guigón-lópez and Gonzalez (2004). The plates were incubated at 26 °C for 6 days with 12 hours of photoperiod under fluorescent white light. After this period, the strains were isolated from green colonies characteristic of the genus and replicated successively to obtain monosporic cultures in a medium of potato dextrose agar (PDA). Identification of the isolates

was at genus level by comparing the vegetative and reproductive structures described by Barnett and Hunter (1998).

2.2.2. Growth rate

From rhizospheric soil of *L. multiflorum* plants was isolated ten strains of *Trichoderma* spp. (denoted TR1, TR2, TR4, TR5, TR6, TR7, TR8, TR9, TR10, and TR11). In order to determine their saprophytic capacity, PDA disc (6 mm) containing the fungal with 7-days old was placed (individually) over PDA at the edge of the plates (9 mm). The *Trichoderma* spp. strains were incubated at 26 °C and 12 hours of photoperiod with fluorescent white light. Radial growth measurements were recorded daily during four days from the edge of the initial inoculum to the extreme area of mycelium development. The growth rate was estimated and obtained in mm day^{-1}. For each fungal isolate was used four replicates (one plate) in a completely randomized design. The experiment was conducted twice.

2.2.3. Dual culture test

The antagonistic capacity of all *Trichoderma* strains was evaluated on the PY2 strain of *P. oryzae* by the dual culture test as described by Rahman et al. (2009). The experiment was performed in a completely randomized design, consisting of 10 treatments, with four replicates for each dual culture. For this, PDA discs (6 mm) containing the fungus (*Trichoderma* spp. or *P. oryzae*) with 7-days old were placed over PDA at the edge of the Petri dish (9 mm). The disc containing *Trichoderma* spp. was placed at the opposing side of the disc containing *P. oryzae,* and the plates were incubated at 26 °C. The radial growth of the *P. oryzae* was recorded daily up to fourth day after inoculation, and the percent inhibition was determined as described by Otadoh et al., (2011):

PI = C–T x 100/ C

Where:

PI = Percent inhibition of mycelial growth,

C = Radial growth of pathogen in control plates (cm),

T = Radial growth of pathogen in dual culture (cm).

Trichoderma spp. efficacy was categorized based on their ability to over grow and inhibit the growth of the *P. oryzae* using the Bell's scale modified by (Cherkupally et al., 2017), where R1 = 100% over growth, R2 = 75% over growth, R3 = 50% over growth, R4 = locked at the point of contact. The experiment was conducted twice

2.2.4. Antibiosis test

The effect of non-volatile and volatile compounds was evaluated by the methods described by Dennis and Webster (1971), Danay et al. (2013). Both antibiosis test was analized under a completely random design, consisisting of 10 treatments and four replicates. For this purpose, a mycelial disk (6 mm) of each strain of *Trichoderma* spp. was deposited in the center of Petri dishes (90 mm) containing PDA covered with a cellophane membrane. Plates containing the mycelial discs were incubated for 36 hours at 26 °C in absolute darkness (until the fungal growth reached more or less ¾ diameter of the plate). At this point, the cellophane membrane was removed. The effect of the non-volatile compounds was evaluated by

depositing a mycelial disc (6 mm) of the PY2 strain of *P. oryzae* on the PDA plate where the cellophane membrane was removed, and then incubated at 26 °C and 12 hours photoperiod with fluorescent white light.

The effect of volatile compounds released by *Trichoderma* spp. over *P. oryzae* was studied using the inverted plate technique (Dennis and Webster, 1971b). A mycelium disk (6 mm) of a 7-days old colony of each strain of *Trichoderma* spp. was transferred individually to plates with PDA medium. The disk was placed in the center of the plate and the cover of each plate was replaced by the bottom of a plate (with PDA medium) containing a mycelial disk of the same size of the PY2 strain. Both bases of the plates were sealed together with parafilm and were incubated four days under the conditions previously described for non-volatile test Plate.

In both antibiosis test, sets without *Trichoderma* spp. were considered as the control treatment. The radial fungal growth was measured at 96 hours after inoculation, using the same formula as in the dual culture tests. The experiment was conducted twice.

2.3. Second phase (in vivo)

This stage aimed to develop a strategy to improve the resistance of *L. multiflorum* plants against *P. oryzae* attack. Initially, the isolate of the *Trichoderma* spp. with the best performance to induce resistance responses, through coating seeds, was selected and identified at specie level. Subsequently, the effect in the resistance of *L. multiflorum* plants against *P. oryzae* of this *Trichoderma* strain and silicon (applied to the soil), used individually or in combination, was evaluated by quantifying the resistance components. Finally, some defense mechanisms associated with the resistance response were determined.

2.3.1. Coated seeds and plant growth

Seeds of *L. multiflorum* cultivar BRS Ponteio were pretreated according to the adapted method of Baseggio et al. (2017). First, seeds underwent an aseptic process with 70% alcohol (1 min) followed by 1% sodium hypochlorite (1 min), three consecutive washes with sterile distilled water and dried under constant air inside a laminar flow. A suspension of 5 mL of 10^7 spores mL^{-1} were prepared from 10-days old colonies for each *Trichoderma* strain. Subsequently, 2.5 g of seeds were subjected to constant rotation (60 rpm) within the spore suspension (according to treatment) at 24 °C during one hour and then were removed and filtered with sterile gauze. The seeds were placed on absorbent paper and incubated at 24 °C under fluorescent light and constant ventilation before being used.

For testing the inoculation quality, one gram of treated seeds was stored at room temperature for 7 days. Subsequently, a total of 100 seeds were placed on 10 Petri dishes (90 mm) containing water-agar (WA), and incubated at 26 °C during 8 days. Then, the percentage of the seeds with *Trichoderma* spp. was determined.

The seeds pretreated with *Trichoderma* strains were sown in 1-liter pots containing clay soil with pH adjusted to 6.5. The seeds were organized in four equidistant points in the square arrangement pot, at each point, two seeds were introduced. Chemical fertilizer 05-20-20 (nitrogen, phosphorus, potassium) was calculated based in total area of pot at rate of 200 kg ha^{-1} at sowing. Total nitrogen (granular urea, N, 46%) input was 200 kg N ha^{-1}, 50% of the total N was applied as basal fertilizer and the remain (50%) at 25 days after seedling emergence.

2.3.2. Plant inoculation and severity assessment

Seventy-day old ryegrass plants were inoculated with a suspension of 1×10^6 conidia mL^{-1} from a 12-days old colony of *P. oryzae* (PY2) growth on oat-agar medium (OA) as is described by da Cruz et al. (2009). After inoculation, the plants were kept in humid chamber with relative humidity (RH) above 90% with the help of a humidifier for 48 hours. Then, plants were kept under greenhouse conditions (26 ± 4 °C; RH ≥80%) until 96 hours after inoculation. The experiment was developed under a completely randomized design consisting of 11 seed treatments (including the control group without treatment) and four replications (1 pot with 4 plants). To obtain the value of each repetition, 10 leaves were sampled completely at random in each pot. The leaves were collected and herborized, then scanned and processed using ImageJ (National Institutes of Health, USA, available in: https://imagej.nih.gov/ij/index.html) to determine the severity and the total number of lesions. The experiment was conducted twice.

2.4. Identification of the antagonist

The isolate with the best ability to control *P. oryzae* according to the evaluation performed *in vitro* and *in vivo* tests was identified, morphologic and molecularly, at the species level. For morphology analysis was used the keys described by Bissett (1992) and the methodology described by (Kubicek and Harman, 2002). Microscopic preparations using clear lactophenol were made from 10-days old colonies. The examinations were performed exclusively under the oil immersion lens through the light microscope (Olympus, mod. CX31) and a LEICA Mod. DMC 2900 camera, and the measurements were made using LAS CORE software (LEICA).

2.4.1. DNA extraction, PCR and sequencing for molecular identification

DNA was extracted from a 12-days old colony using a fungal DNA isolation kit (NORGEN) according to the manufacturer recommendations. PCR was performed using the primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify part of the rDNA 18s gene, the internal transcribed spacer 1 (ITS1), the 5.8S gene, ITS2 and part of the 26s gene (White et al., 1990), the primer pairs LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR05 (5'-TCCTGAGGGAAACTTCG-3') for amplify part of the large ribosomal subunit region (Brown et al., 2014; Vilgalys and Hester, 1990), the primers EF983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and EF2218R (5'-ATGACACCRACRGCRACRGTYTG -3') for amplify the elongation factor (EF-1α) (Schoch et al., 2009) and the primer set CAL-228F (5'-GAGTTCAAGGAGGCCTTCTCCC-3') and CAL-2Rd (5'-TGRTCNGCCTCDCGGATCATCTC-3') to amplify part of the calmodulin gene (CAL) (Carbone and Kohn, 1999; Groenewald et al., 2013). The PCR programs were carried out according to the specifications made by White et al. (1990) for the ITS, Manamgoda et al. (2012) for the LSU and EF-1α regions, and Groenewald et al. (2013) for the CAL. PCR products were purified with a PCR products purification Kit (MEBEP BIOSCENCE) according to the manufacturer's instructions.

2.4.2. Sequencing and phylogenetic analysis

The PCR fragments were sequenced in both directions using the same primers used for PCR amplification and the BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, USA), following the manufacture´s recommendations. The products were analyzed on an ABI Prism 3730 XL DNA Sequencer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences in the MEGA 7 (Molecular Evolutionary Genetics Analyses) (Kumar et al., 2016) and were edited manually when necessary. To determine preliminary identification the TrichOKEY 2.0 tool (available in http://www.isth.info/tools/molkey/index.php) was used to analyze and compared with the ITS region 1 and 2, and, Megablast for the sequences of the LSU, EF1-α and CAL region. Phylogenetic analysis was made from 38 isolates of the Genbank database (including the isolate LIPP TR10 derived in this study and the outgroup) belonging to 12 species of *Trichoderma* (Table 1) and was performed by the combined the ITS and EF1-α sequences. Each loci were aligned using MAFFT v. 7 (Katoh et al., 2019). The alignments (ITS and EF1-α) were trimmed in the MEGA 7 when necessary and phylogenetic analyses of sequence data were done in Phylogenetic Analysis Using Parsimony (PAUP; version 4.0b10; Sinauer Associates, Sunderland, MA) (Swofford, 2002). The individual alignments of different loci were subsequently concatenated with the Fasta alignment joiner tool of Fabox ver. 1.5 available in https://users-birc.au.dk/~palle/php/fabox/index.php. *Hypomyces virescens* (G A i1899) served as an external group for phylogenetic analyzes of *Trichoderma* species. Phylogenetic reconstructions of individual and concatenated trees were performed by selecting the best fit model, using the Akaike Information Criteria, in which the "GTR+I+G" model was selected for both alignments. The Bayesian analysis was performed using the Mrbayes on XSEDE tool in CIPRES Science Gateway platform and an algorithm of Markov Chain Monte Carlo (MCMC) was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.2.7a (Ronquist et al., 2012). The analyses MCMC were run from random trees for 10 000 000 generations and sampled every 1000 generations. 25% of trees were discarded as the burn-in phase of the analysis and the remaining trees were used to calculate subsequent posterior probabilities (PP). The trees were reviewed and adjusted in Geneious 9.0.5 and customized in Adobe Illustrator CS5.1. The sequences have been lodged at GenBank and the alignment and trees in the TreeBASE (www.treebase.org/treebase-web/home.html).

Table 1
Details of *Trichoderma* species subjected to multi-gene DNA sequence analysis.

Species	Strain no.[1]	Source	Location	GenBank accession numbers[2]		Reference
				ITS	EF-1α	
Hypomyces virescens	G.A. i1899, CBS 127161	*Schizophyllum commune*	Cuba, Camaguey	FN859453	FN868771	Põldmaa, 2011
T. aggressivum	TRS27	Mushroom farm, air	Poland, Sierakowice	KP009302	KP008994	Oskiera et al., 2015
T. atrobrunneum	TRS60	Mushroom compost	Poland, Balcerów	KP009260	KP008874	Oskiera et al., 2015
	TRS86	Soil	Poland, Rzeczyce	KP009254	KP008876	Oskiera et al., 2015
	TRS91	Soil	Poland, Wysoka	KP009261	KP008875	Oskiera et al., 2015
T. atroviride	LIPP TR10	Rhizosphere (*Lolium multiflorum*)	Brazil, Rio Grande do Sul	To be added	To be added	This study
	TRS5	Mushroom compost	Poland, Łódź	KJ786741	KJ786822	Oskiera et al., 2015
	TRS6	Mushroom compost	Poland, Sława	KJ786750	KJ786831	Oskiera et al., 2015
	TRS17	Mushroom compost	Poland, Skierniewice	KJ786734	KJ786815	Oskiera et al., 2015
	TRS18	Mushroom compost	Poland, Maków	KJ786757	KJ786839	Oskiera et al., 2015
	TRS26	Soil	Poland, Lipniak near Kock	KJ786751	KJ786832	Oskiera et al., 2015
	TRS28	Mushroom farm, floor	Poland, Kolonia Bolimowska	KJ786721	KJ786802	Oskiera et al., 2015
	TRS30	Soil	Poland, Skierniewice	KJ786720	KJ786801	Oskiera et al., 2015
	TRS31	Soil	Poland, Balcerów	KJ786740	KJ786821	Oskiera et al., 2015
	TRS32	Mushroom farm, air	Poland, Maków	KJ786744	KJ786825	Oskiera et al., 2015
T. crassum	TRS113	Soil	Poland, Wola Skromowska	KP009300	KP008865	Oskiera et al., 2015
T. gamsii	TRS123	Soil	Poland, Strobów	KP009330	KP008922	Oskiera et al., 2015
	TRS125	Soil	Poland, Wola Skromowska	KP009326	KP008919	Oskiera et al., 2015
T. hamatum	DAOM 233483 , CIB T144	Rhizosphere (*Phaseolus*)	Colombia, Antioquia	EU280105	EU279959	Hoyos-Carvajal et al., 2009
	TRS121	Forest wood	Poland, Głuchów	KP009342	KP008953	Oskiera et al., 2015
	TRS127	Unknown	Poland, Unkanown	KP009343	KP008954	Oskiera et al., 2015
T. lentiforme	TRS35x	Soil	Poland, Lękosz	KP009231	KP008899	Oskiera et al., 2015
	TRS58x	Soil	Poland, Wadowice	KP009256	KP008901	Oskiera et al., 2015
	TRS63x	Soil	Poland, Skierniewice	KP009230	KP008898	Oskiera et al., 2015
	TRS64	Unknown	Poland, Pszczyna	KP009229	KP008897	Oskiera et al., 2015
	TRS65x	Unknown	Poland, Sokołow Podlaski	KP009228	KP008896	Oskiera et al., 2015

T. pleuroticola	TRS70	Forest wood	Poland, Głuchów	KP009264	KP008951	Oskiera et al., 2015
	TRS120	Forest wood	Poland, Głuchów	KP009265	KP008952	Oskiera et al., 2015
T. simmonsii	TRS66	Unknown	Poland, Parczew	KP009222	KP008867	Oskiera et al., 2015
	TRS67	Unknown	Poland, Radzyń Podlaski	KP009221	KP008866	Oskiera et al., 2015
	TRS75	Mushroom farm	Poland, Sierakowice	KP009223	KP008871	Oskiera et al., 2015
	TRS77	Mushroom farm, floor	Poland, Maków	KP009226	KP008869	Oskiera et al., 2015
	TRS80	Mushroom compost	Poland, Skierniewice	KP009225	KP008868	Oskiera et al., 2015
	TRS85MO	Mushroom farm, air	Poland, Sierakowice	KP009224	KP008872	Oskiera et al., 2015
T. spirale	TRS111	Soil	Poland, Rudzieniec	KP009301	KP008963	Oskiera et al., 2015
T. virens	TRS106	Mushroom compost	Poland, LMCc	KP009291	KP008854	Oskiera et al., 2015
	TRS112	Mushroom compost	Poland, Skierniewice	KP009296	KP008860	Oskiera et al., 2015
T. viridescens	TRS35	Soil	Poland, Lękosz	KP009338	KP008930	Oskiera et al., 2015

[1]TRS: Department of Vegetable Crops Protection, Research Institute of Horticulture, Poland; CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; DAOM: Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; LIPP: Laboratório de Interação Planta Patógeno, RS, Brazil.

[2]ITS: internal transcribed spacers intervening 5.8S nrDNA; EF-1α: partial translation elongation factor 1-alpha gene.

2.5. Evaluation of resistance components and biochemical defenses in plants treated with Trichoderma atroviride and silicon

2.5.1. Experimental conditions

The physic-chemical characteristics of the soil used were as follow: 190 g kg^{-1} of clay; pH $CaCl_2$ (0.01 M) 5.1; Na = 10, P (Mehlich) 9.4 and K (Mehlich) 47 mg dm^{-3}, whereas Al^{3+} (0.2), Ca^{2+} (4.2), Mg^{2+} (0.9), and CTC (8.1) $cmol_c$ dm^{-3}. Besides, base saturation and Al^{+3} saturation were 65 and 3.5%, respectively and organic matter 17.9 g dm^{-3}. The ratios Ca/Mg, Ca/k and Mg/K were 4.57, 35.00 and 7.50, respectively. The concentration of available Si (extracted with 0.01 M $CaCl_2$) was 6.0 ppm determined according to Korndörfer et al. (2004). Soil fertility was adjusted as described by Dorneles et al. (2018). Calcium silicate, a source of silicon, calcium and magnesium, or extra-fine limestone, a source of calcium and magnesium, was added in the soil at rate of 7.8 ton ha^{-1} to increase soil pH to 6.5. Calcium and magnesium were equalized among treatments using calcium carbonate and magnesium carbonate, respectively. Soil correctives were incorporate 30 days before sowing.

2.5.2. Coated seeds with Trichoderma spp. strains

Seeds of *L. multiflorum* cv. BRS Ponteio were pretreated with the strain TR10 of *Trichoderma atroviride* as described previously.

2.5.3. Experimental design

The experiment was conducted in greenhouse conditions (26 ± 4 °C and 80% RH) with a pH adjustment of 6.5 in all treatments as previously described in the section 2.5.1, and, was organized under a completely random design in two-way factorial consisting of four management types [1- Soil amended with 7.8 ton ha^{-1} of extra-fine limestone and seed pretreatment with 10^7 of spores mL^{-1} of *Trichoderma atroviride* (strain TR10) (T), 2-Soil amended with of 7.8 ton ha^{-1} of calcium silicate in the soil (Si), 3)- Soil amended with of 7.8 ton ha^{-1} of calcium silicate in the soil and seed pretreatment with 10^7 of spores mL^{-1} of *T. atroviride* (TSi) and 4)- the control treatment, soil amended with 7.8 ton ha^{-1} of extra-fine limestone (TE)] and two inoculation [1- plants inoculated with 1×10^5 spores mL^{-1} of *P. oryzae* or 2- plants sprayed with water (mock inoculated)]. For each treatment was used four replicates and each replicate was constituted by one pot with four plants. The experiment was conducted twice.

2.5.4. Resistance components evaluation

To evaluate the resistance of *L. multiflorum* against *P. oryzae*, several resistance components were assessed as described by Dallagnol et al. (2011). Briefly, the relative efficacy of the infection (RIE) was calculated from the percentage of conidia that managed to establish stable parasitic relationships in *L. multiflorum* and cause lesions. The incubation period (IP) was time, in hours, between the inoculation with *P. oryzae* and the manifestation of gray leaf spot symptoms. The latent period (LP) was defined by the time in hours, between the plant inoculation with *P. oryzae* and the start of conidia production in the formed lesion. The size of the lesion (SL) was obtained from the final average size of the lesions after its expansion ceases, the the lesion expansion rate (LER) was obtained from plotting the lesion size as a function of the time. The final severity (SEV) and the number of lesions per unit of leaf area in cm^2 (NL) was evalutated 10 days after the inoculation following the procedures described previously.

2.6. Enzymes assays

Approximately five leaves of *L. multiflorum* plants per replication of each management type (TE, T, Si, and TSi) were collected at 24, 48, 72, and 96 hours after the inoculation with *P. oryzae*. The samples were flash-frozen with N_2 and then stored in a freezer (-80 °C) until they were analyzed. The same procedure was used to sampling leaves from plants sprayed with water. The crude extract was obtained from 1.0 g of leaf tissue according methodology described in Dallagnol et al. (2015). The peroxidase activity (POX, EC 1.11.1.7), was determined by colorimetric quantification of the oxidation of pyrogallol (Sigma-Aldrich) and was expressed as moles of purpurogallin produced min^{-1} mg^{-1} of protein, using the extinction coefficient of 2.47 mM cm^{-1}. The superoxide dismutase (SOD, EC 1.15.1.1) was determined by quantifying the photoreduction of nitroblue tetrazolium (NBT) following Gupta et al. (1993). A unit of SOD was defined as the amount of enzyme needed to inhibit 50% of the photoreduction of NBT, expressing the results in units of SOD mg^{-1} protein. Catalase activity (CAT, EC 1.11.1.6) was calculated based on the quantification of the degradation of hydrogen peroxide (H_2O_2) (MERK) (Azevedo et al., 1998). The results were expressed in μmol of degraded H_2O_2 min^{-1} mg^{-1} protein. The activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was quantified colorimetrically by the formation of trans-cinnamic acid from phenylalanine (Sigma-Aldrich) and was expressed as μmol of trans-cinnamic acid produced per min^{-1} mg^{-1} of protein (Guerra et al., 2013). The protein concentration used for enzyme activity calculation was obtained by the method of Bradford, using bovine serum albumin as the standard (Bradford, 1976). The protein and enzymatic determinations were performed in a spectrophotometer (model UV-UM51- Bel®).

2.7. Silicon concentration in soil and leaf tissues

Twelve days after the inoculation with *P. oryzae*, samples of leaf and soil, from each treatment, were collected to determined silicon concentration. The percentage of silicon in the leaves was determined from the yellow method with sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2), while the soil content was obtained with the blue method, using calcium chloride ($CaCl_2$) as extractor base, and was expressed in mg of Si kg^{-1} according to the methodology described by Korndörfer et al.(2004).

2.8. Statistical analyses

The results of each experiment were individually analyzed and submitted to analyses of variance and when significant, the Tukey test ($P < 0.05$) was applied. The Pearson linear correlation technique was used to determine the relationships among the main resistance components evaluated (RIE, LER, LS, NL, and SEV) and the LP and PI were excluded due to the null correlation given by similarity of the results among treatments. The results of the enzyme assay were used to calculate the area under progress curve of the enzyme activity using an adaptation of the formula of Shaner and Finney (1977). The analysis was performed using the Statistical Analysis System (Cary, North Carolina) SAS statistical package (version 9.4), and the graphics were designed in Graphad prism (version 8).

3. Results

3.1. First phase (in vitro)

3.1.2. Growth rate

The analysis of variance (ANOVA) of the growth rate (GR) showed significant differences (P <0.0001) among strains. The GR varied between 16 and 24 mm per day (Fig. 1, A). TR2 and TR6 obtained the fastest GR (24 mm day^{-1}), however, the value was statistically similar to those obtained by TR1, TR4, and TR5. The lowest value of GR (16 mm day^{-1}) was obtained with TR8, which did not differ from TR7, TR9, and TR10.

3.1.3. Dual culture test

The ANOVA of the dual test showed significant differences (P = 0.0079). The inhibition ranged from 30 (TR1) to 39% (TR7) (Fig. 1, B), and these values were statistically different between them, but not of the rest of the strains. Regarding the type of antagonism, six strains (TR1, TR4, TR5, TR6, TR7, and TR9) were characterized by the type of R4 antagonism, that is, they stoped the growth at the point where they did contact with the *P. oryzae* colony. Two strains (TR2 and TR11) showed the type R3, in which the antagonist overgrown on the colony of *P. oryzae* by 50%, and TR8 and TR10 in the type R1, because 100% of the *P. oryzae* colony was colonized in all replications.

3.1.4. Antibiosis test

In the antibiosis test for non-volatile compounds (Fig. 1, C), a significant effect was observed (P <0.0001). Two main groups were formed for the percentage of inhibition; one comprising the isolates TR2, TR4, TR5, and TR6 showing a range of inhibition from 42 to 52%, and the second comprising the isolates TR1, TR7, TR8, TR9, and TR10 which varied from 13 to 25% of inhibition. In the case of volatile compounds (Fig. 2, D), significant differences were also observed (P = 0.0009), although with a smaller proportion of the inhibition, ranging between 11 and 23%. The highest value of inhibition was reached by the strain TR10 which was different statistically of the TR9 (11%) and TR11 (14%).

3.2. Second phase (in vivo)

3.2.1. Coated seeds

The seeds of *L. multiflorum*, at eighth day of incubation in agar-water at 26 C, showed colonization by *Trichoderma* spp. between 96 and 100%. The strains TR1, TR2, TR4, TR8, and TR10 obtained 100%, TR5 and TR7 with 99%, while TR6, TR9, and TR11 reached 98, 97 and 96%, respectively. The non-inoculated seeds did not present any visible colonies of *Trichoderma* spp.

3.2.2. Severity assessment

The severity of gray leaf spot, at 96 hours after of inoculation, showed significant differences (P<0.0001) among *Trichoderma* strains used to coat the seeds (Fig. 2, A). Control treatment without inoculation (TES) showed the highest severity (18%), a value that did not differ statistically from those reached on plants from seeds coated with the strains TR7, TR6, TR5. The lower severity occurred on plants from seeds coated with TR10 (5%), which was similar to that observed on plants from seeds treated with TR4 (8%), TR8 (7%), TR9 (7%) and

TR1 (6%). For the number of lesions (Fig. 2, B) occurred a similar pattern as for severity, being influenced significantly (P <0.0001) by the *Trichoderma* strain that coated the seeds. However, only plants from seeds treated with TR1, TR8, and TR10 showed significantly reduction in the number of lesions compared to the TES (11 lesions).

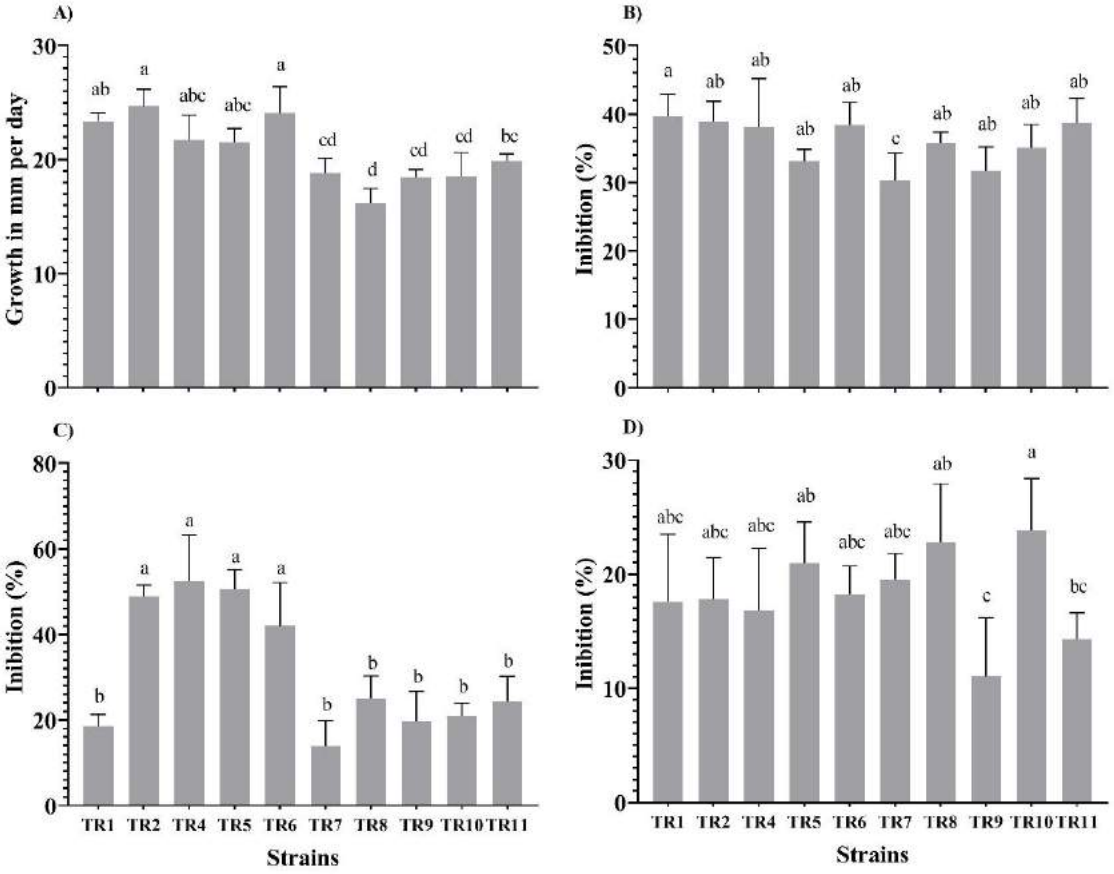

Fig. 1. Growth and antagonistic activity of *Trichoderma* spp. (isolated from the rhizosphere of *Lolium multiflorum* plants) on the pathogen, *Pyricularia oryzae* in potato dextrose agar (PDA). A= Growth of *Trichoderma* strains in PDA. B= Percentage of inhibition of *Pyricularia oryzae* in paired colonies, C= Percentage of inhibition in the test of cellophane paper, for non-volatile compounds and D= Percentage of inhibition in the overlaid plates for volatile compounds. The error bars represent the standard deviation of the means and different letters indicate significant difference (P < 0.05) according to Tukey test.

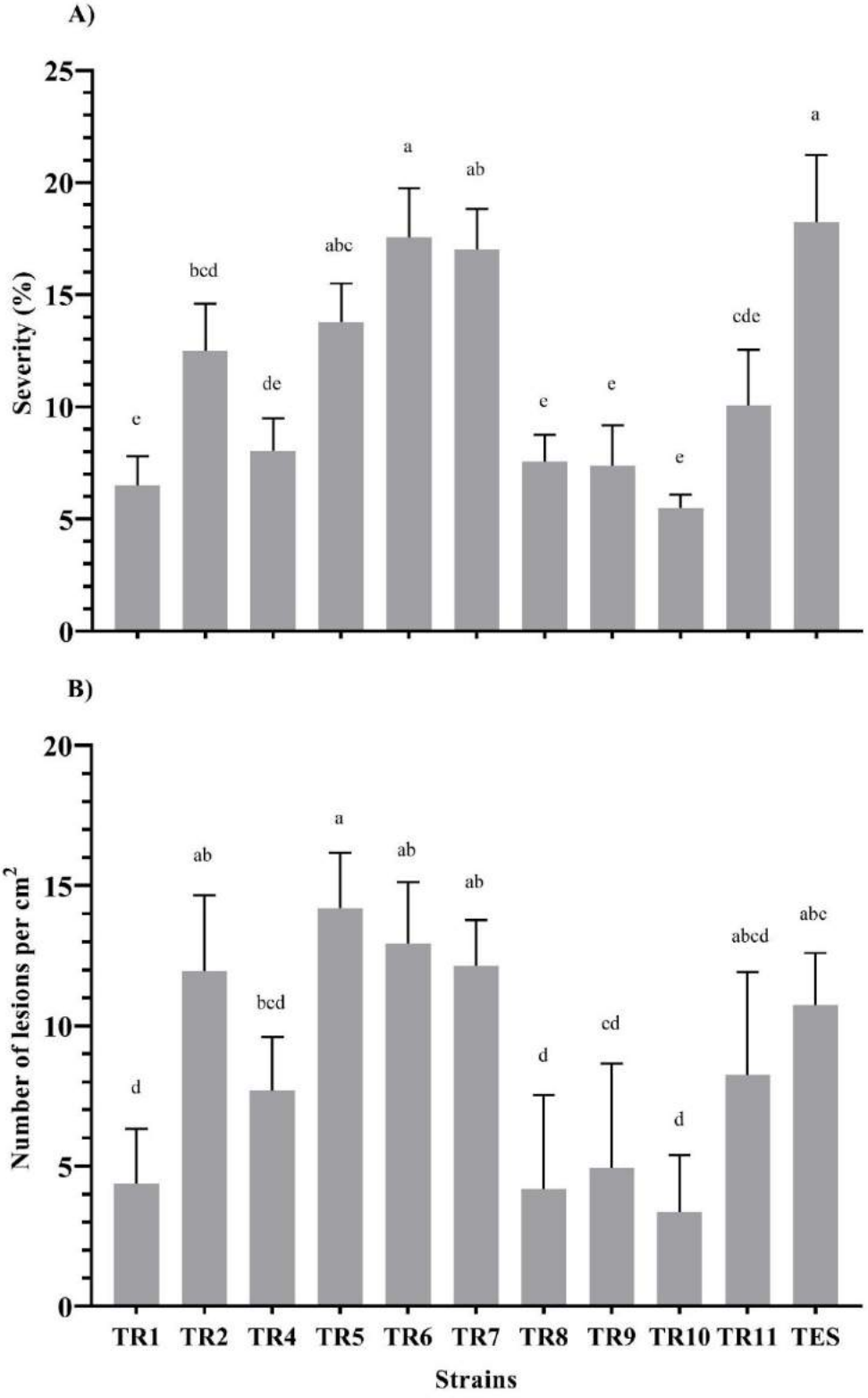

Fig. 2. Disease intensity on *Lolium multiflorum* plants (70 days old) cv. Ponteio, pretreated (in the seeds) with different strains of *Trichoderma* spp. isolated from the rhizosphere. A= Final severity of gray leaf spot and B= Number of lesions per cm^2 of leaf area at 96 h after inoculation with $1x10^6$ spores mL^{-1} of PY2 strain of *Pyricularia oryzae*. The error bars represent the standard deviation of the means and different letters indicate significant difference ($P < 0.05$) according to Tukey test.

3.3. Morphology

The morphological descriptions of the strain TR10 coincided mostly with descriptions of *Trichoderma atroviride* P. Karsten (Bissett, 1992). The colony grown rapidly (6-8 cm) after 4 days, at 20 ± 1°C on 2% malt extract agar (MEA) according to criteria used by (Kubicek and Harman, 2002), varying from pale green to dark green, yellowish color (reverse), giving off a coconut-like aroma. On MEA (2%) the mycelium growing mostly submerged, translucent with concentering rings, the surface was granular and with abundant sporulation, on PDA (Fig. 3A) mycelium was floccose, white, with irregular pustules, effuse sporulation, covering the entire plate, while on water agar, the mycelium was translucent and superficial, with poor sporulation, and showing irregular pustules from white to green (Fig. 3B). The conidiophores were mostly flexuous, with primary branches emerging at regular intervals (Fig. 3D), generally paired of three (usually short). Production of abundant round shaped chlamydospores (5.4-12.9 µm) (Fig 3E). Solitary phialide, sometimes whorled, lageniform, often curved, 4.4–11.3 × 2.2–3.3 µm. Subglobose to short ellipsoidal conidia (Fig. 3C), varying in color from light green to dark green (when mature) with smooth walls, 2.6–3.9 × 2.2–3.4 µm.

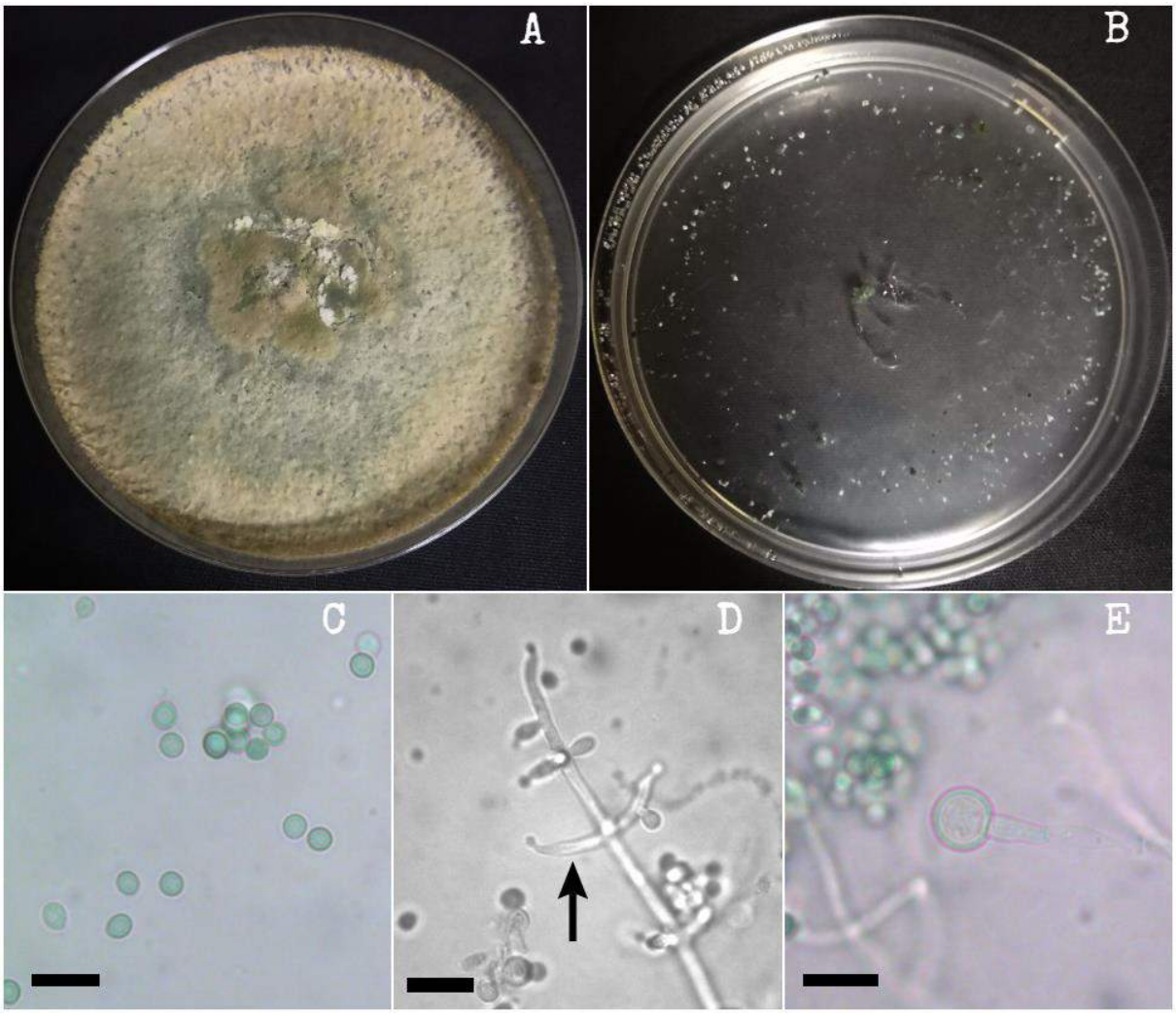

Fig. 3. Microscopic morphology of *Trichoderma atroviride* (strain LIPP TR10) from a 10-day colony grown on potato dextrose agar medium (a) and water agar medium (b); conidia (c); conidiophore, phialides and conidias (d); clamydosphore (e); langeniforme phialide (black arrow). – Scale bars; c-e =10 µm.

3.4. Molecular identification

Preliminary identifications with the TrichOKEY 2.0 tool from the ITS region showed that isolated TR10 was similar to *Trichoderma atroviride* with high identification reliability. Also, Megablast searches of the LSU, EF-1 and CAL sequences showed 99.24 (LSU), 99.79 (EF-1) and 99.55% (CAL) identity with the same species, with access numbers KM099499, XM_014085582, and DQ122166, respectively.

The dataset consisted of 1190 characters, representing 38 taxa, including the outgroup, being 570 for EF-1 and 620 for ITS. The alignment included 140 unique site patterns, being 72 for ITS and 68 for EF-1. After topological convergence of the Bayesian runs, 60008 trees were generated and 15002 subsequently sampled (using a burn-in fraction of 0.25) in order to generate the Bayesian phylogeny. The resulting phylogenetic tree (Fig. 4) showed consistent clustering of all taxa over each one of the trees (data not shown). Bayesian posterior probabilities (PP) are presented on the left of each node. The polyphasic approach used in this study revealed irrefutably that isolate TR10 belongs to the *Trichoderma atroviride* clade, alongside with strains isolated from soil and mushroom compost.

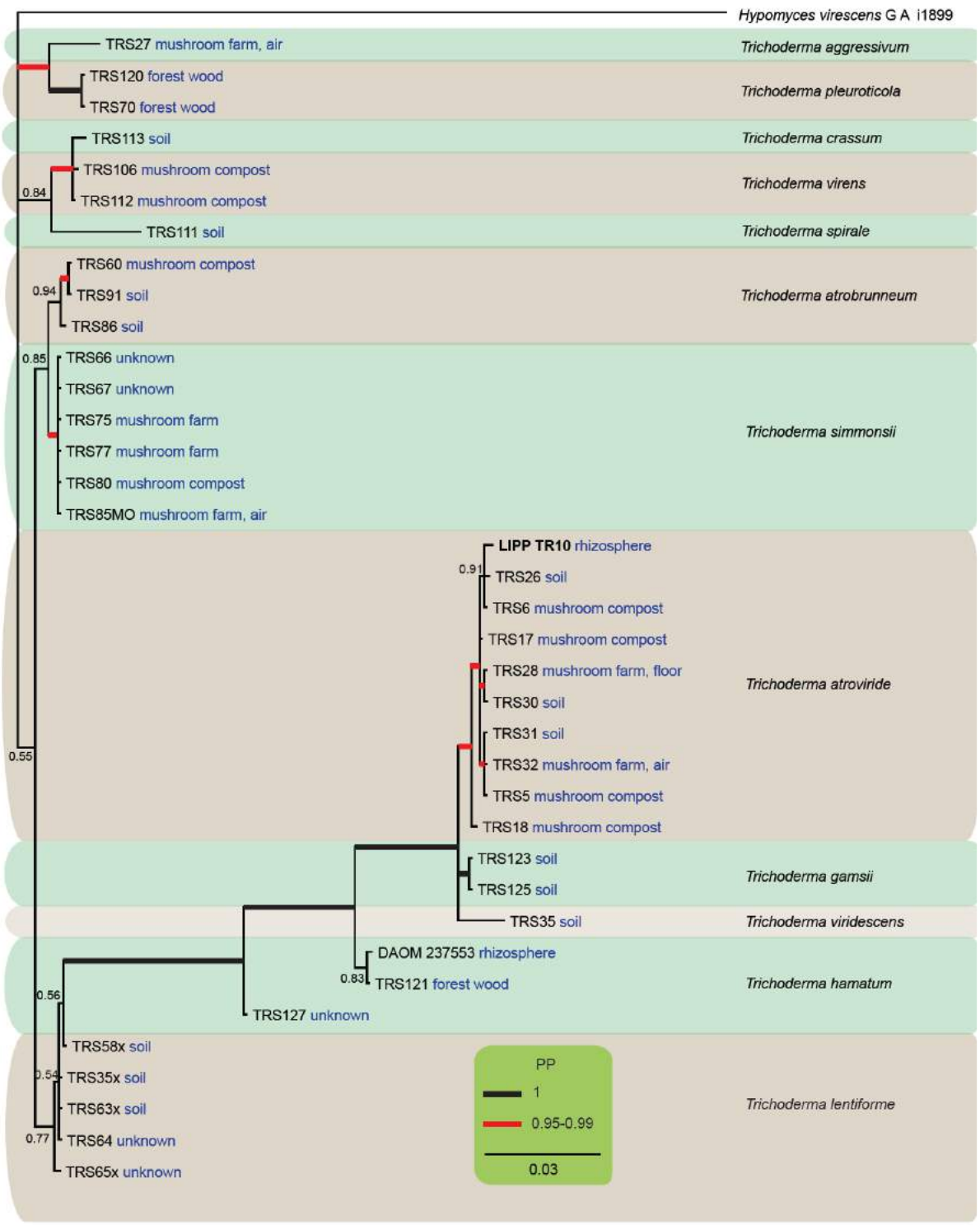

Fig. 4. Consensus phylogram (50 % majority rule) of *Trichoderma* species, from a Bayesian analysis of the combined 2-gene sequence alignment (ITS and EF-1). Bayesian posterior probabilities are indicated with color-coded branches and numbers (see legend). The scale bar indicates 0.03 expected changes per site. Isolate from Brazil is indicated in bold. Substrates are indicated in blue text. The tree was rooted to *Hypomyces virescens* (isolate CBS GA i1 1899).

3.5. Resistance components

The number of lesions (Fig. 5, A), the lesion expansion rate (Fig. 5, C), the relative infection efficiency (Fig. 5, D), and gray leaf spot severity (Fig. 5, E) were significantly reduced by Si, T, and TSi compared to TE.

Only for the severity occurred difference among the three treatments (Si, T and TSi), reducing the variable in 58, 52 and 42% for Si, T and TSi, compared to the TE. The incubation period was not affected by treatments, because the disease symptoms appeared around 94 hours after inoculation, regardless of the treatment. The same results occurred for the latent period which ranged from 106 to 110 hours without significant differences among treatments (data not shown).

The correlation data showed a strong relationship between the variables (Fig. 5, F), mainly between LER and RIE ($r = 0.99$), RIE with NL ($r = 0.98$), and NL with LER ($r = 0.97$). While the SEV also showed positive correlation although lower than the previous ones with LER ($r = 0.89$) and RIE ($r = 0.87$), however, the variable with lower correlation rates was the LS which reached its maximum ($r = 0.79$) with NL.

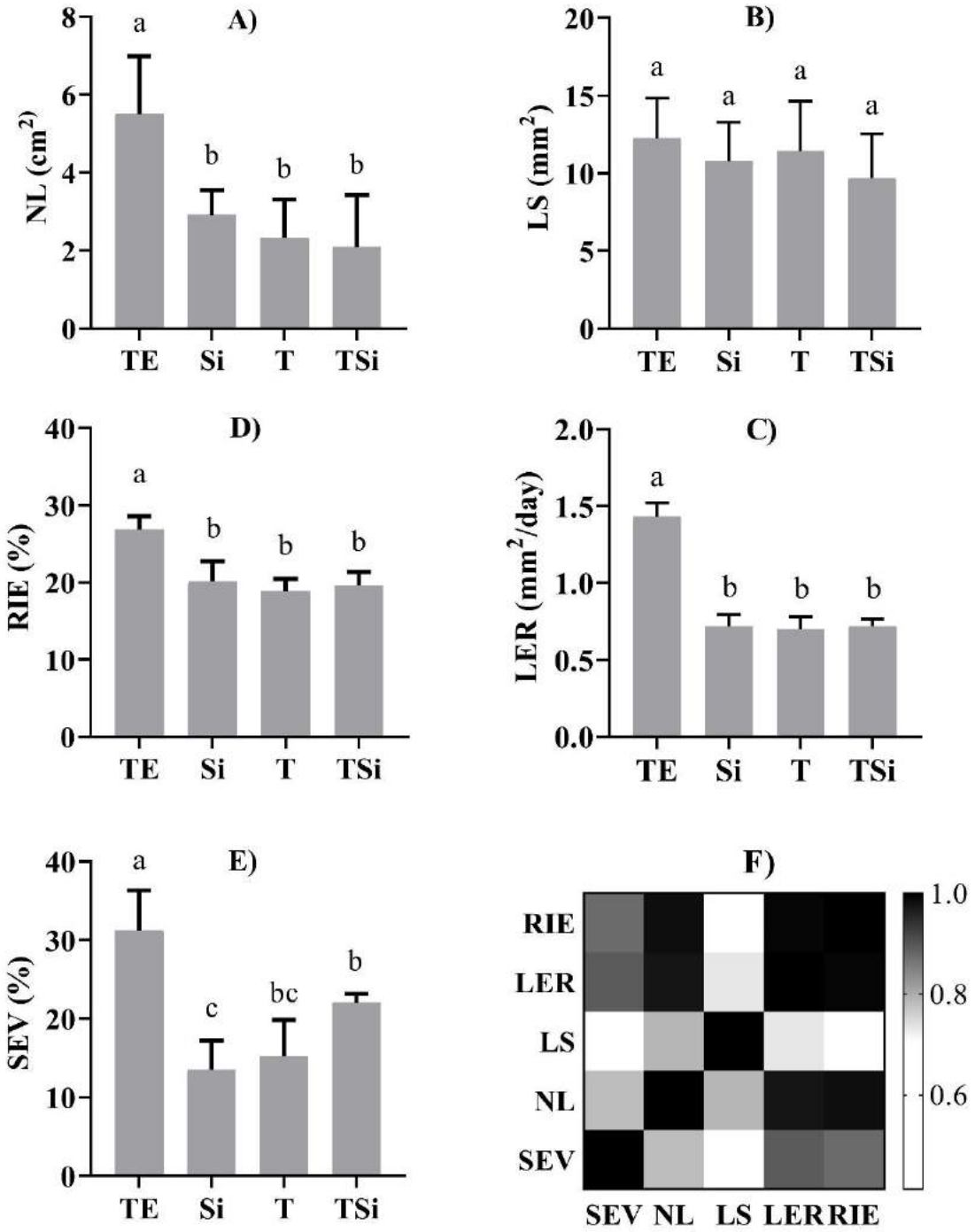

Fig. 5. Resistance components of Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio inoculated with $1x10^6$ mL^{-1} spores of *Pyricularia oryzae*. TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} extra-fine limestone, Si = soil modified with 7.8 ton ha-1 of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with $6x10^8$ spores mL^{-1} of *Trichoderma atroviride* (TR10). TSi =T and Si combined. A) Number of lesions (NL) per cm² of leaf area, B) lesion size (LS), C) lesion expansion rate (LER), D) relative infection efficiency (RIE), E) final severity of gray leaf spot at 10 days after inoculation (SEV) and F) heat map of the correlation indices (Pearson) of the resistance components. The error bars represent the standard deviation of the means. The different letter indicates significant differences (P <0.05%) according to the Tukey test.

Table 2

Analysis of variance of management type (MT), plant inoculation (PI) and sampling time (ST) on the activities of the enzymes superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and phenylalanine ammonia-lyase (PAL).

Source of variation	Df	*F* values[1]			
		SOD	POX	CAT	PAL
MT	3	0.49ns	92.34*	35.37*	108.09*
PI	1	15.36*	511.29*	39.81*	398.53*
ST	3	196.85*	26.04*	5.31ns	65.57*
MT× PI	3	2.6ns	35.5*	9.84*	88.29*
MT × ST	9	1.5ns	8.46*	6.69*	20.42*
PI× ST	3	4.88*	6.16*	19.15*	27.61*
MT × PI × ST	9	3.13*	7.67*	3.58*	28.18*

[1] Levels of probability: ns = not significant, and * = 0.05.

3.6. *Enzyme assays*

The analysis of variance showed that the management type (MT) of coated seeds and/or incorporation of silicon in the soil (T, Si or TSi), did not affect the SOD activity in *L. multiflorum* plants (Table 2), since that treatments not inoculated (Fig. 6, A) behaved similarly to those inoculated with *P. oryzae* (Fig. 6, B), and the area under the progress curve of the enzyme activity (AUPCEA) showed no significant variation (Fig. 6, C).

CAT activity showed significant differences (P <0.0001) due to MT, plant inoculation (PI), or interaction of the factors (Table 2). In the inoculated plants (Fig. 6, D), the T, TSi, and Si treatment showed greater activity (34% more activity) compared to TE at 72 hours after inoculation (hai), while at 96 hai only TSi contrasted with TE, because the activity in Si and T decreased considerably. This difference is easily observed with the high AUPCEA in plants inoculated with *P. oryzae* (PI), and also for plants treated with *T. atroviride* (Fig. 6, F).

POX activity also showed significant differences (P <0.0001) regardless of the source of variation or interaction between factors (Table 2). In inoculated plants (Fig. 6, H), the activity was significantly higher for T and TSi from 24 to 72 hai, compared to TE. The Si treatment only showed a slight difference (15% more activity) from TE at 24 hai, however at 48 hai even less activity was evidenced, meanwhile at 72 and 96 hai, Si and TE showed a similar pattern. The AUPCEA (Fig. 6, I) confirmed that only the treatments with *T. atroviride* showed POX activity significantly higher in comparison to TE.

PAL activity also showed significant differences (P <0.0001) in all sources of variation and their interaction (Table 2). At 24 and 48 hai the T, Si, and TSi contrasted significantly with TE, however, the highest PAL activity (> 60%) was recorded at 72 hai with T (Fig. 6, K) and it was significantly contrasting (P <0.0001) with all treatments. At 96 hai, PAL activity was partially reduced in T, however, it continued to statistically contrast with Si, TE, and TSi. At this time, TSi had a mild response, but sufficient to obtain differences with TE. Cocerning to analisys of AUPCEA (Fig. 6 L), the hiher value was observed to T treatment, but the Si and TSi also showed values higher than TE.

3.7. *Silicon concentration*

The silicon concentration in the soil at the end of the experiment in treatments Si (5.3 mg Si kg^{-1} of dry soil) and TSi (5.1 mg Si kg^{-1} of dry soil) showed 30% more silicon than in treatments without application, T (3.1 mg Si kg^{-1}) and TE (3.3 mg Si kg^{-1}). This fact directly influenced the concentration of this element in the leaves of the plant, because it increased 3 times the concentration with silicon (30 g Si kg^{-1} of dry leaf tissue) about the treatments without (10 g Si kg^{-1} of dry leaf tissue).

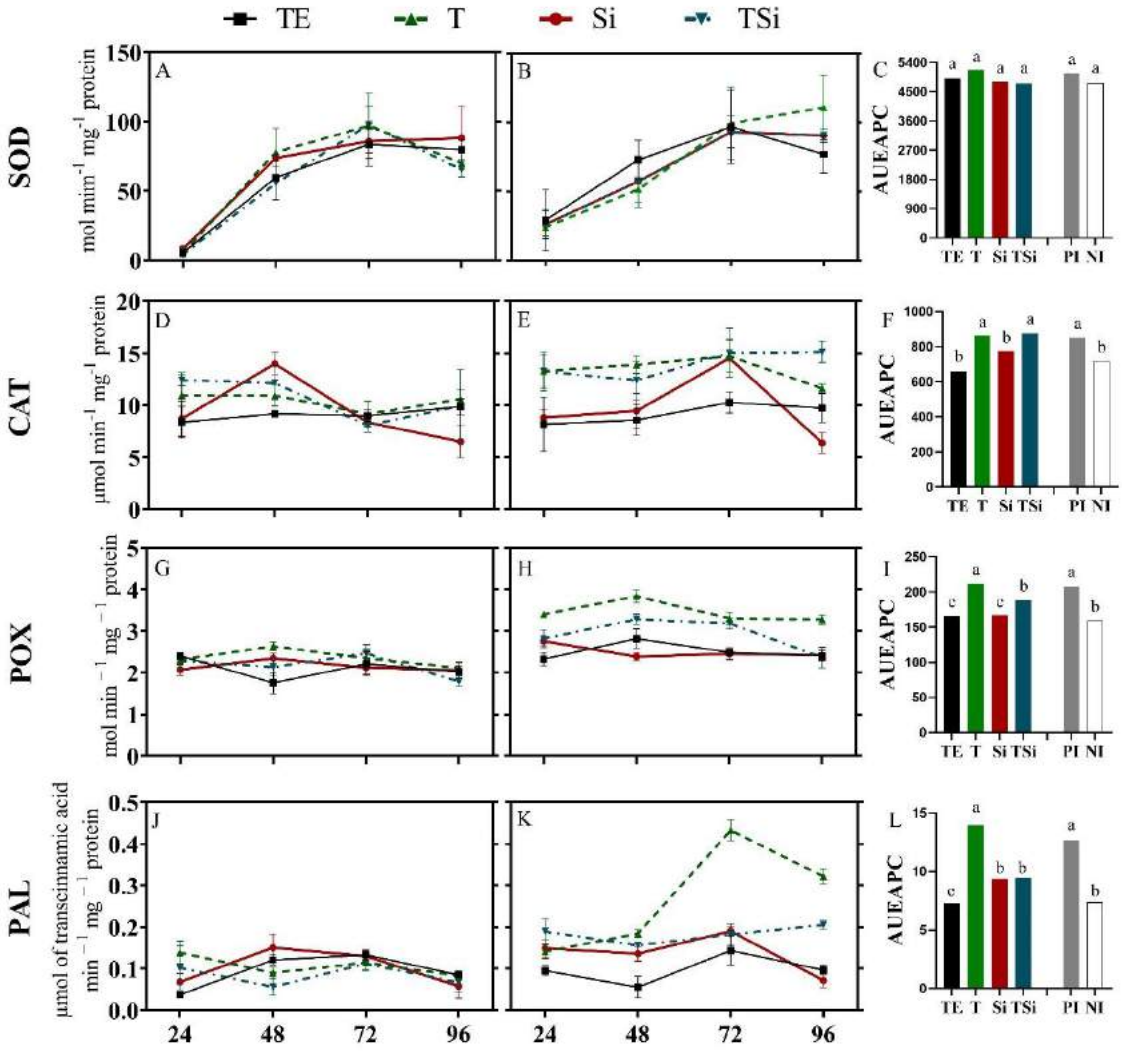

Fig. 6. Enzymatic activities in leaves of Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio. TE= Control treatment, plants grown under soil with pH adjusted (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si= soil amended with 7.8 ton ha^{-1} calcium silicate (pH 6.5), T= soil pH adjusted like TE treatment and seeds pre-treated with $6x10^8$ spores mL^{-1} of *Trichoderma atroviride* (TR10). TSi= soil pH adjusted like Si treatment and seeds pre-treated with $6x10^8$ spores mL^{-1} of *T. atroviride* (TR10). SOD= Superoxide dismutase, CAT= Catalase, POX= Peroxidase, PAL= Phenylalanine ammonia-lyase. Not inoculated (A, D, G, and J) and inoculated plants (B, E, H, and K) with $1x10^6$ spores mL^{-1} of *Pyricularia oryzae*. Comparison of the area under the progress curve of the enzyme activity (AUPCEA) on leaves of plants inoculated with *P. oryzae* for TE, T, Si and TSi, and between plants inoculated with *P. oryzae* (PI) not inoculated (NI)= not inoculated ((C, F, I, and L). Each point on trend lines represents the mean of four replications. Vertical lines in each point represent the standard derivation. The different letter indicates significant differences (P <0.05%) according to the Tukey test.

4. Discussion

The purpose of this study was to evaluate whether the severity of the gray leaf spot (caused by *P. oryzae*) in *L. multiflorum* plants could be reduced by incorporating silicon in the soil, coating seeds with *Trichoderma* (native) or both treatments at from resistance induction. The *Trichoderma atroviride* strain TR10 showed great performance in the *in vitro* and *in vivo* tests, being selected for experimental tests under conditions of a greenhouse. TR10 showed the ability (test of dual cultures) to inhibit the growth of *P. oryzae*, and it was also characterized by having a type of R1 antagonism due the colony of pathogen was completily overgrowth by the antagonist. Furthermore, this strain of *T. atroviride* showed great inhibition potential from volatile compounds. These evidences shown that TR10 is a promising microorganism to be used on the control of *P. oryzae* causing the gray leaf spot in *L. multiflorum.* Similar results were also reported previously to different *Trichoderma* isolates have shown the capacity to inhibit aggressive pathogens such as *Fusarium oxysporum* f. sp. *phaseoli*, *Fusarium oxysporum* f. sp. *melongenae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* by volatile and non-volatile compounds, released during the confrontation (Amin et al., 2010; Cherkupally et al., 2017; Otadoh et al., 2011). Although *P.* oryzae infects shoot, the direct or indirect biological control mechanisms of the antagonist used for seed coating are still important because the antagonist may reduce the source of the inoculum associated to the seeds. The reduction of the inoculum may increases the percentage of seeds germination, the vigor of the seedlings, and also reduces the transmission of *P. oryzae* from seeds to seedlings, how is already known occur in other crops such as rice (Manandhar et al., 1998).

The induction of resistance by seed inoculation has been little studied, however, species such as *T. harzianum* and *T. viride* have shown inhibition percentages greater than 60% when compared against *P. oryzae* (*in vitro*), and a similar reduction (between 51.3 and 48.5%) of the blast severity caused by this pathogen in *Oryza sativa* L. plants when the seeds were treated with these antagonists. (Kumar et al., 2019). Furthermore, the efficiency of *T. harzianum* (strain T-22) improves protection against *Pythium ultimum* in tomato plants (*Solanum lycopersicum*) cv. Jubilee when were inoculated on seeds (Mastouri et al., 2010), and also with another isolate of *T. harzianum* (T-1055) used for sunflower (*Helianthus annuus*) seeds treatment, improved protection against the pathogen *R. solani* through increased host defense responses (B. N. Singh et al., 2014). However, there is no evidence of the use of *Trichoderma* for seeds treatment in *L. multiflorum* to improve resistance to pathogens. In this sense, one of the findings of this research was to identify bio-controllers with the ability to stimulate resistance in *L. multiflorum* and results of this study showed consistency that TR10 could be used as a potential biocontrol agent.

In the *in vivo* tests, five *Trichoderma* strains (TR1, TR4, TR8, TR9, and TR10) reduced the gray leaf spot severity on *L. multiflorum*, of which TR10 obtained the best performance because it accumulated the lowest value of disease severity and the number of lesions (70% less than TE treatment). Indirect evaluation of resistance in the host showed similarity in effects mediated by treatment with silicon, *T. atroviride* and the combination of both, as demonstrated by the variables of number of lesions, lesion expansion rate and relative infection efficiency. There is evidence that the use of silicon promote a response similar to the use of *Trichoderma* sp. and *Bacillus subtillis* as a seeds treatment to reduce the severity and area under the curve of disease progress in soybean plants attacked by *Fusarium* sp. (Rodrigues et al., 2017). Similar results were found in this study, where treatment with silicon and *T. atroviride* obtained an

effect of equal magnitude about the reduction in severity of the gray leaf spot, however in the combination TSi no additional advantage was observed. Furthermore, the combination of soluble silicon and *T. harzianum* did not exert an effect additive on the *in vitro* growth of *P. aphanidermatum* (Rachniyom and Jaenaksorn, 2008), which suggests that these treatments have better performance used in isolation than in association, however, more evidence is required to support this idea.

On the other hand, the resistance components revealed a strong relationship that indicated the dependency between the variables, however, the lesion size showed the least association and no significant difference between the treatments. This was caused because the lesion stopped expanding at different times, but in the end, they reached the same size, which could underestimate the efficacy of the treatments, since the main effect may be related to time (delay in colonization for example) as is evidenced indirectly with LER, notwithstanding, LS showed that in successful *P. oryzae* infections the treatments used are not effective, that is, there is no decrease in the affected area after establishment. In this sense, the final severity (SEV) was mainly affected by NL, LER and RIE.

The CAT, POX, and PAL activities were stimulated in the 3 treatments evaluated (Si, T, and TSi) explaining partially the results of the resistance components. The Si mainly boosted CAT activity at 72 hours (30% in relation to control) and peroxidase activity at 24 and 48 hours. The CAT provides the cell with an energy-efficient mechanism to eliminate hydrogen peroxide (H_2O_2) when the cells are stressed by an energy decompensation (caused by the attack of pathogens for example). H_2O_2 is rapidly generated through emergency catabolic processes, and this molecule is degraded by CAT without consuming reducing cellular equivalents (Mhamdi et al., 2010). While POX are glycoproteins of the plant kingdom present in various forms of isoenzymes, which confer lignification, suberization, wound healing, general stress response and protection against pathogen attack (Saraiva et al., 2010). Silicon has been shown to potentiate the activity of the CAT enzyme in the pathosystem *Oryza sativa-P. oryzae* (Domiciano et al., 2015), and of POX in *Lolium perenne-Magnaphorte oryzae* (Rahman et al., 2015). However, the present study found that *T. atroviride* offers a greater enzymatic response (CAT, POX and PAL) in relation to Si, mainly with PAL at 72 hours after inoculation. PAL catalyzes the first step of the general phenylpropanoid pathway and this enzyme activity can be stimulated by some physical and chemical factors such as the deposition of extracellular compounds excreted by fungi (Bolwell et al., 1985; Halpin, 2004). The enzymatic reaction produces cinnamic acid, which acts as a precursor to many secondary substances, including lignin, flavonoid pigments, coumarins, salicylic acid, and phytoalexins (Chen and McClure, 2000; Halpin, 2004; Smith-Becker et al., 1998). It is well known that the activity of the PAL enzyme in plants can be induced by the action of *Trichoderma* species. For example, seeds treated with *T. hamatum* from pearl millet induced resistance against downy mildew caused by *Sclerospora graminicola*, and part of this effect was conferred by the PAL (Nayaka et al., 2017). Likewise, PAL was stimulated together with the expression of the AcPR1 and AcPAL1 genes, which indicated an initial activation of the salicylic acid-dependent defense pathways by *T. asperellum* when it was inoculated in *Allium cepa* infected by *Sclerotium cepivorum* (causal agent of white rot disease) resulting in lower incidence and mortality of plants in greenhouse and field (Rivera-Méndez et al., 2020).

The concentration of silicon in the plant tissue revealed that the application of 7.8 ton ha^{-1} of calcium silicate in the soil increased the element concentration in leaves by 300%. This

fact could explain the gray leaf spot reduction on plants from Si treatment, as demonstrated in the study by Rahman et al. (2015) with *L. perenne-M. oryzae* pathosystem, in which they observed a physical effect in plants supplied with silicon related to the callose deposition at 48 and 72 ha of pathogen. The physical barrier mediated by silicon is also formed from the translocation of this element from the root to the air part through the transport stream in the xylem, consequently, silicon accumulates in the epidermal tissue in the form of a thin layer of silicon-cellulose membrane and is associated with pectin and calcium ions, in this way, the double cuticular layer can mechanically protect and strengthen plant structures (Meena et al., 2014). Thus, silicon can improve the resistance of plants from the formation of a physical barrier of silicified epidermal cells, by forming papillae, double cuticular layer or through a thick layer of silica under the cuticle (Samuels et al., 1994; Sun et al., 2010). The reinforcement of the wall from the application of silicon in rice plants has shown benefits by reducing the number of penetration sites and the severity of *P. oryzae* (Kim et al., 2002; Sousa et al., 2013). In this sense, results of the resistance components support the idea of the formation of a physical barrier mediated by silicon, since it reduces the number of lesions, at the same time there is a delay in the rate of colonization in cases where it was successful. However, our results support also the hypothesis that biochemical responses can be stimulated by silicon in *L. multiflorum*, besides, it has been proven that PAL, lipoxygenase, phenolic compounds and flavonoids have been stimulated in *L. perenne* plants when infected with *M. oryzae* (Rahman et al., 2015). Likewise, in the *M. oryzae-Oryza sativa* pathosystem, the stimulation of phytoalexin production has been evidenced, in which silicon has an active and complex role (Rodrigues et al., 2004). In this sense, biochemical and molecular analysis and histological studies could provide better understanding of the mechanisms by which silicon increased resistance of ryegrass.

In conclusion, this study provides two alternatives (mediated by *T. atroviride* and silicon) to reduce the severity of gray leaf spot, improving the resistance of the plant from a sustainable strategy. This is the first report of *T. atroviride* associated with *L. multiflorum* in Brazil. To proper identify its potential as a biocontrol agent against *P. oryzae*, further studies in the field in grazing systems are needed.

Declaration of Competing Interest

The authors involved in this work declare that they do not have any type of financial interest, competitive order, or personal relationship that has influenced the work reported in this paper.

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Silicon, pretreatment of seeds with *Trichoderma atroviride*, and cutoff frequency reduce the intensity of leaf spots on *Lolium multiflorum*

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ABSTRACT

Italian ryegrass has become one of the most widely used plants for cattle fooding in southern Brazil, especially during the winter period, due to availability, nutritional qualities and the ease agronomic management. However, the production of biomass and seeds, important for natural reestablishment of the crop, are threatened by pathogens. The objective of this work was to determine the level of importance of leaf spot diseases occurring naturally in *Lolium multiflorum*, develop sustainable control strategies, and evaluate their effectiveness. For this, during 2018 and 2019 field seasons, it was evaluated the effect of seeds treatment with *Trichoderma atroviride*, soil amended with silicon, or both, in combination with different cutoff frequencies (2, 1 and 0 cuts) on the intensity of leaf diseases. The main diseases recorded were the gray leaf spot (*Pyricularia oryzae*), brown leaf spot (*Bipolaris sorokiniana*) and parrot's eye leaf spot (*Cercospora lolii*), in order of importance, and they reached an incidence of 100, 60 and 35% and severity of 27, 15, and 8%, respectively. The treatments carried out in the soil and/or in the seeds reduced the area under the disease curve progress of the gray leaf spot (from 31 to 45%) and of the brown leaf spot (from 38 to 65%). Also, both diseases, gray spot and brown leaf spot, decreased by about 65% when the cuts frequency increased to 2 cuts. In conclusion, our work reveals three different strategies to reduce the intensity of leaf spots diseases in *L. multiflorum*; the cutoff frequency, the pretreatment of seeds with *T. atroviride*, and the incorporation of silicon in the soil. This is the first evidence of sustainable disease management in this forage crop.

Keywords

Winter forage, disease control, management strategies, integrated disease management, sustainable biomass production

1 Introduction

The Italian ryegrass (*Lolium multiflorum* L.) has become one of the most important plants in the livestock production sector due to nutritional advantages (mainly high protein value), easy establishment and availability of food during winter, which is a period of shortage (Hoffmann et al. 2014). In 2017, this activity included a total effective of 173 million heads and 30 billion liters of milk, of which 6.6% of cattle and 13% of milk was produced in the state of Rio Grande do Sul (IBGE 2020). The feeding of this cattle in this region is mainly based on grasslands of natural origin and cultivated, which occupy 35 and 8%, respectively, of the total area available for agricultural establishments (IBGE 2020).

Pasture production is currently limited by factors such as agricultural crop expansion, desertification, improper management, climatic factors and biotic problems (pests and diseases). Among biotic problems, fungal diseases have been noted for significantly reducing the amount of forage and seed production in the southern region of Brazil (Dias-filho 2017).

There are currently no studies showing which are the main pathogens, damage scales or management strategies to counteract losses (in quantity and quality) of fodder and seeds. Also, chemical control is not possible, because in Brazil, until now, there is no fungicide registered for use on *L. multiflorum*. In this sense, alternatives with lower environmental impact, such as cultural practices aiming to reduce the source of inoculum, the incorporation of silicon (Si) in the soil and the use of biological control with fungi such as *Trichoderma* spp, are expected to be useful.

In the case of Si, despite being considered a non-essential element (Takahashi et al. 1990), it increases the defense of plants trough physical and biochemical mechanisms (Debona et al. 2017). The physical mechanism, occur due to the formation of the Si-cuticle double layer, cell wall strengthening, and the formation of papillae, restricting or delaying the entrance and the spread of fungal hyphae in the host tissues (Kim et al. 2002; Cai et al. 2008; Sousa et al. 2013; Domiciano et al. 2013). Furthermore of physical mechanism, Si also promotes changes in the transcription of genes related to the defense of the plant, resulting in up or down regulate defense enzymes, biosynthesis and accumulation of antimicrobial compounds (phenylpropanoids, flavonoids, phytoalexins, and pathogenesis-related proteins) and alteration in salicylic, jasmonic and ethylene signaling pathways (Fortunato et al. 2015; Rahman et al. 2015; Dorneles et al. 2018).

Trichoderma spp. can also affect pathogens by acting, in a direct and or indirect modes, such as competition for space and nutrients, mycoparasitism, antibiosis, and resistance induction (Harman et al. 2004). Resistance induction promoted by *Trichoderma* spp. shares mechanisms quite similar to those observed in Si-treated plants, such as the formation of papillae and callose deposition (Nayaka et al.

2017), the stimulation of defense enzymes such as phenylalanine ammonium lyase, chitinase, peroxidase, and biosynthesis and accumulation of phytoalexins and PR proteins (Singh et al. 2018).

Furthermore, the removal of the infected material from the field, through grazing or mechanical cutting, also could reduce the disease intensity (Nunes and Mittelmann 2009). Cutting management has been indicated in forage crops such as *Dactylis glomerata* for the control of foliar pathogens such as *Drechslera dactylidis* (Braverman et al. 1986). In this sense, the frequent cutting is an option for to control of foliar diseases in forage grasses (Fig. 1), because restricts build-up disease by removing the source of secondary inoculum.

Considering all the above, the objective of this study was to evaluate the intensity and level of importance of foliar diseases, occurring naturally, on *L. multiflorum*, and to evaluate the efficacy of different management systems based on the reduction of the inoculum source (frequence of pasture cutting), incorporation of silicon in the soil, and pretreatment of seeds with *Trichoderma atroviride* on the control of pathogens causing leaf spots.

Fig. 1. Forage cutting frequency in *Lolium multiflorum* plants (four months old) for foliar disease management in Rio Grande do Sul, Brazil. On the left side, a plot with plants that received two cuts and on the right the control treatment without cutting.

2 Materials and methods

2.1 Study area

The experiment was conducted at the Agriculture Center of Palma (31°48'06.4"S 52°30'18.6"W) belonging to Federal University of Pelotas. The experiment was conducted in two successive field seasons of 2018 and 2019 under soil classified as Red-Yellow Argisol, with sandy loam texture. The physicochemical conditions of the soil, in the experimental area, were the following: 190 g kg^{-1} of clay; pH $CaCl_2$ (0.01 M) 5.1; Na and P (Mehlich) = 10 and 9.4 mg dm^3, respectively, K (Mehlich), Al^{3+}, Ca^{2+}, Mg^{2+} and CTC = 47, 0.2, 4.2, 0.9 and 8.1 $cmol_c$ dm^{-3}, respectively. Besides, the soil had 65% base saturation and 3.5% Al^{+3} saturation, organic matter with 17.9 g dm-^{3}. Ca/Mg, Ca/k and Mg/K molar ratios had 4.57, 35.00 and 7.50, respectively, while the available silicon (extracted with 0.01 M $CaCl_2$) of 0.54 mg Si g^{-1}.

2.2 Experimental design

The study was developed under a random block design in two-way factorial defined in a 4×3 arrangement, consisting of four treatments (1 - application of silicon source (Si) in the soil, 2 - seeds inoculated with *Trichoderma atroviride* (strain, TR10) (T), 3 - application of Si in the soil and seeds inoculated with *T. atroviride* (TSi) and 4 - control group (TE)), and three grass cutoff frequencies (1 - two cuts (2C), 2 - one cut (1C) and 3 - no cut) with four replications. Each experimental plot had 2 × 5 m, and they were separated by 1 m and a 30 cm deep furrow to prevent runoff of rainwater from one plot to the other. The total area of the experiment was 980 m² and was carried out twice in the same area in order to evaluate the residual effect of the treatments.

2.3 Seeds inoculation with *T. atroviride*

The seeds used in the treatments 2 and 3 (as described in section 2.2) were inoculated with *T. atroviride* (strain TR10). For this, the antagonist was grown per 10 days on potato-dextrose-agar at 25° C and 12-hour photoperiod. Conidia were collected in sterile water and the concentration was adjusted to 6×10^8 conidia mL^{-1}. Subsequently, 1250 grams of seeds of ryegrass were immersed in 4 L of the conidia suspension and kept in a rotary shaker (60 rpm) at 24 ° C during 1 hour. Then, seeds were removed from the suspension by filtering with sterile gauze, and kept by 24 hours on blotting paper (to remove excess moisture) at 24ºC and constant ventilation. In order to avoid differences in the water content in the seeds, treatments 1 and 4 were treated with water without the TR10 strain, following the same procedure.

2.4 Agronomic management

The herbicide glyphosate (Roundup®, 3.0 L ha^{-1} of c.p.) was used to eliminate weeds in the experimental area. At 20 days after weeds control, the soil of the experimental area was ploughed and harrowed using a tractor. Calcium silicate (source of silicon, calcium and magnesium) or extra-fine lime

(source of calcium and magnesium), which their composition was described in Dorneles et al (2018), were used to increase the soil pH to 6.5. In the crop season of 2018, calcium silicate and extra-fine limestone were incorporate in the soil at rate of 7.8 t ha^{-1} by harrowing, 30 days before sowing. In the crop season 2019, the correctives (calcium silicate and extra-fine limestone) at rate of 1 t ha^{-1} were hand applied on the soil surface without removing it. Seeds of the ryegrass (*Lolium multiflorum*) from cultivar BRS Ponteio (Embrapa®), were sowed (7 rows, 0.13 m rows spacing) with a mechanical seeder (SEMEATO®, mod. SHP 249) at density of 25 kg ha^{-1} of seeds. Chemical fertilizer 05-20-20 (nitrogen, phosphorus, potassium) at rate of 200 kg ha^{-1} was applied at sowing. Total nitrogen (granular urea, N, 46%) input was 200 kg N ha^{-1} yr^{-1}, 50% of the total N was applied as a basal fertilizer; the remaining (50%) was top-dressed in equally amount, at 25 days after seedling emergence and just after the first cutting. Methylsulfuron (HERBEX®; 6 g c.p. ha^{-1}) was used to control the weeds, as necessary to ensure these factors would not influence the outcome of the experiment. The sowing in the crop season 2018 was realized at 05/22/2018, and in 2019 it occurred by natural regeneration.

2.4.1 Cutting of the ryegrass

The cuts on ryegrass was applied when plants were 30 cm high. The cutting was conducted with a mower, at a height of 10 cm at ground level. The period between each cut was 37 days. At the end of each cut, the plant debris were removed from the experimental area.

2.5 Disease assessment

Disease intensity was measured through quantifying the incidence and severity. Nine and eighteen evaluations were performed during the crop season 2018 (from August 17 to October 26) and 2019 (from May 10 to September 13), respectively. Evaluations started shortly after seedlings emergence and repeated at 7-day intervals until the end of the crop cycle. The incidence was determinate on 40 leaves, while the severity was done from 10 leaves per plot (randomly sampled). After collecting, the leaves were herborized and dried into bond paper sheets. The disease incidence was visually quantified by verifying the plants (symptomatic or not) and it was jointly determined which pathogen was associated to the lesion. Thus, after the evaluation, the percentage of leaves affected by each pathogen was estimated. The disease severity was quantified by visual assessing the percentage of the affected area by each disease concerning the total area of the leaf. All evaluations were performed by same person from the beginning to end of the experiment in both crop seasons. Data were expressed as a percentage of tissue affected by each disease. The data of disease severity over time were used for determination of the area under the disease progress curve (AUDPC) according to Shaner and Finney (1977).

2.6 Determination of silicon concentrations in soil and plant tissues

The concentration of silicon (Si) in soil was determinate by the blue method through calcium chloride. Soil samples were randomly collected into each experimental plot from 0-20 cm deep, at beginning and at the end of the experiment. The samples from the four plots of each treatment were pooled for Si

determination. Each soil sample was dried in a forced-air oven at 45 °C until constant weight and after was ground using a mortar and pistil. In a 150 mL plastic bottle, ten grams of the ground soil was placed 100 mL of calcium chloride ($CaCl_2.2\ H_2O$) 0.01 mol L^{-1} and kept on a horizontal shaker at 50 rpm for one hour. Then, the solution was repositioned to the settling of solid residues and finally filtered with a gauze. The filtrate was again repositioned for 20 hours to decant the residues that remained after filtration. After this period 10 mL of the sample was placed in another 50 mL vial. Then 1 mL of the sulfur-molybdenum solution [$(NH4)_6Mo_7O_{24}.4H_2O$] 75 g L^{-1}, sulfuric acid (H_2SO_4) 18 N (500 g L^{-1}) was added with a pipette, stirred gently and the reaction was maintained at room temperature for 10 minutes. Then 2 mL of the 200 mg L^{-1} tartaric acid solution was added, after 5 minutes 10 mL of the ascorbic acid solution was added and one hour after the reaction was read on a UV-Visible spectrophotometer at 660 nm. The regression curve was performed from silicon standards (1000 mg L^{-1}) adjusted to different concentrations (0.4, 1.0, and 2.0 mg L^{-1} Si): a 10 mL aliquot of each standard was used (instead of filtrate) following the same process for the samples reactions and adding the same quantities of reagents and concentrations. The results were expressed in milligrams of Si per kilogram of dry soil (KORNDÖRFER, et al. 2004).

The concentration of Si in leaf tissue was determined by colorimetric analysis (Yellow method) from 0.1 g of alkaline-digested dry tissue. Leaf tissue samples were collected at the end of the experiment and then dried in a forced-air oven at 65 °C until constant weight. Then was ground in a Thomas Willey mill (Thomas Scientific, Swedesboro, NJ) using a screen of 40 mesh. After, 0.1 g of the ground vegetable material was placed into a 50 mL polypropylene tube in which was added and mixed (into the lab protect cabinet) 2 ml of hydrogen peroxide (H_2O_2) 500 g L^{-1} and 3 ml sodium hydroxide (NaOH) 500 g L^{-1}. Then, tubes were placed in the water bath (95 °C) for approximately 1 hour, about fifteen times with a 5 second vortex agitation period, until the release of gases in the samples was no longer observed. Afterward, the tubes were capped and placed inside the autoclave (1h at 123 ° C and 1.5 atm). After digestion, 45 mL of distilled water was added and after one-hour decantation of non-alkalinized substances, 2 mL of the extract was collected and transferred to a 50 mL plastic container. A volume of 23 mL was completed from 2 mL of the extract, 17 ml of distilled water, 2 mL of hydrochloric acid (HCI) 500 g L^{-1} and 2 mL of ammonium molybdate [$(NH_4)6Mo_7O_{24}.4H_2O$] 100 g L^{-1}, pH 8 (adjusted with NaOH 400 g L^{-1}). The mixture was gently shaken for 2 minutes and allowed to stand for 8 min until the yellow color appeared (in the samples containing Si). After, 2 mL of oxalic acid [$(COOH)2.\ 2H_2O$] 75 g L^{-1} was added, shaking slightly the solution, and after 2 min, the absorbance determined in a spectrophotometer at 410 nm. The regression curve was performed from standard Si content solutions (0, 2, 4, 6 and 8 mg L^{-1}). 19 mL of each Si standard solution was completed at a final volume of 25 mL with the same reagent quantities and concentrations used for the samples. Results were expressed in miligrams of Si per kilogram of dry leaf tissue (KORNDÖRFER, et al. 2004).

2.7 Data analysis

The severity values were used to calculate the AUDPC for each disease evaluated in the two years of experiment. The generated AUDPC data was tested using the Shapiro Wilk test to assess the normality of the variations. Later they were submitted to analysis of variance (ANOVA) and when it was significant, the Tukey test was applied ($P < 0.05\%$). The analysis was performed using the SAS program (version 9.4, SAS Institute, Inc. Cary, NC, USA) and the graphics were designed in Graphad prism (version 8, California corp, USA).

3 Results

3.1 Season 2018

3.1.1 Incidence

In the 2018 season was recorded the occurrence of gray leaf spot (*Pyricularia oryzae*), brown leaf spot (*Bipolaris sorokiniana*) and parrot's eye leaf spot (*Cercospora lolii*). The incidence of these diseases increased over time, reaching in greater amount at late of crop cycle, however, being decreased by treatment and/or cuts (Fig. 2). For gray leaf spot, the incidence showed rapid increase from October 5 (Fig. 2, A-C). Plants from the treatments (TE, T and Si) exempt from cutting showed incidence around 80%, while in plants from TSi the value was around 50%, a reduction of 30% compared to the maximum values of incidence (Fig. 2, A). Plants that received one cut (Fig. 2, B) the incidence was similar among TSi and TE treatments (higher than 80%); however, the T treatment stood out from the other treatments reducing disease incidence. In plants with 2 cuts (Fig. 2, C), the incidence reached up to 27.5%, in the final of crop cycle, being a little bit lower in TSi. For gray leaf spot, in general, one cut not affected the disease incidence, except for TSi that showed increase in the disease incidence, but two cuts reduced in more than 50% the disease incidence regardless of the treatment.

The incidence of brown leaf spot (Fig. 2, D-F) rose rapidly, but a week later compared to gray leaf spot, at the time the plant entered the flowering stage. Plants from TE, T and Si treatments without cut (Fig. 2, D) accumulated a value of 30% in the final evaluation, being 8% higher than TSi (22%). As shown in Fig. 2 (E), when a cut was applied, the T (55%) and TE (60%) treatments accumulated similar incidence values, which contrasted with Si and TSi that obtained 25 and 12%, respectively. However, the application of 2 cuts proved to be the best strategy for the management of the disease (Fig. 2, F), because at the end of the experiment no plants infected with *B. sorokiniana* were found regardless of treatment with Si and/or *T. atroviride*.

The majority of plants with parrot's eye leaf spot were found in treatments where the cut was not applied (Fig. 2, G), particularly at the end of flowering. At this stage, the disease incidence in the TE and T reached 25%, while Si reached 35% and TSi 30%. The effect of single cut (Fig. 2, H) was less effective to Si (27.5%) and T (15%), while higher effect occurred for TE (5%) and TSi (2.5%) that accumulated

the lowest values. However, as in brown leaf spot, the effect of 2 cuts (Fig. 2, H) was decisive to eliminate the presence of *Cercospora* sp. in the final evaluation regardless of the treatmen.

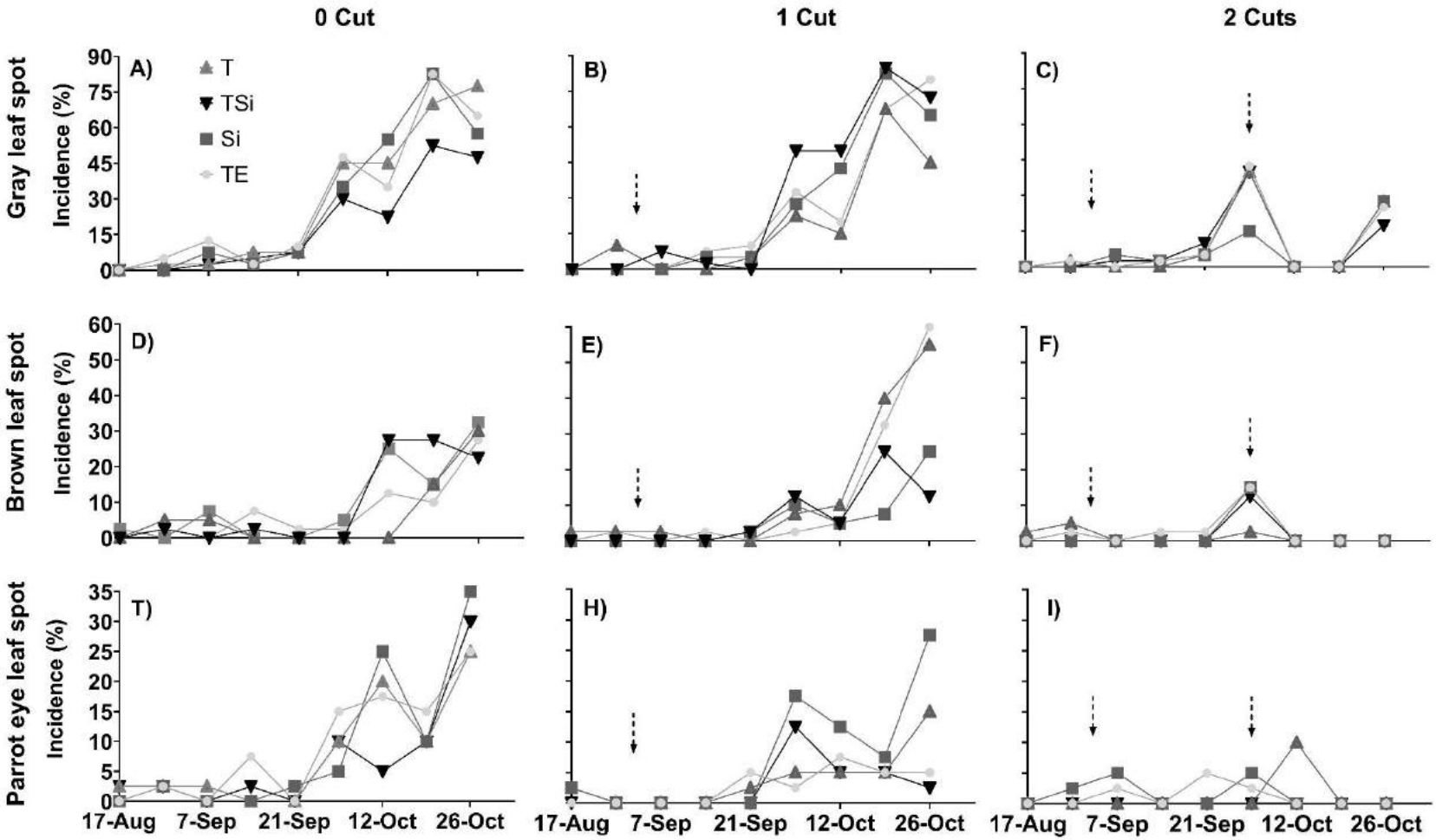

Fig. 2. Incidence of leaf spot diseases (season 2018) on Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio, under different soil management, seeds inoculation, and cutoff frequency. TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si = soil modified with 7.8 ton ha^{-1} of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with $6x10^8$ spores mL^{-1} of *Trichoderma atroviride* (TR10), and TSi = soil modified with 7.8 ton ha^{-1} of calcium silicate and seeds pretreated with $6x10^8$ spores mL^{-1} of *T. atroviride*. Black arrow indicates the time of cut.

3.1.2 Severity

The severity of the diseases, in the 2018 season, followed a pattern similar to that shown with the incidence, varying with the treatment and number of cuts performed. In the case of gray leaf spot (Fig. 3, A-C), an increase in the severity occurred from October 5. As of this date, in plants without cut (Fig. 3, A), the disease increased faster in the control (TE) reaching to the maximum severity value, 13.5%, at the last evaluation. On the other hand, the progress of gray leaf spot was delayed by treatments, especially by TSi, but at the last evaluation reached to the value of 12% to TSi, 10% to T and 9% to Si. Plants that received one cut (Fig. 3, B), negative effects were observed in the control treatment, which increased the severity up to 16% in the final evaluation, and TSi which obtained values between 4 and 9% in evaluations after the cutting, reaching a final severity of 12%. However, plants from Si and T treatment were less affected tending to reduce the disease severity after cutting. For two cuts, (Fig. 3, C), plants from TE showed an increase in the severity, but in the last evaluation the gray leaf spot severity was lower than 3% regardless of treatment.

The severity of brown leaf spot varied mainly due to the cutting treatment (Fig. 3, D-F). In plants without cut (Fig. 3, D), the brown leaf spot reached to the maximum severity value (6%) on the TSi in mid-October, however, in the last evaluation the severity was similar among treatments (less than 4%). When performed one cut (Fig. 3, E), the disease severity reached to 15.5% in the final evaluation on plants of the TE, while the less values of the severity of brown leaf spot was achieved with T (3%), TSi (1%) and Si (2%). On the other hand, when two cuts were made (Fig. 3, F) there was no diseases at the last evaluation.

The severity of parrot's eye leaf spot had a pattern similar to brown leaf spot being affected mainly by the number of cuts performed (Fig. 3, G-I). Plants without cut (Fig. 3, D), reached to the higher severity value on TE and TSi, while the lower value occurred on plants from Si and T treatments. When one cut was made (Fig. 3, H), the severity remained less than 2.5% in all treatments and evaluations performed. The same tendency was observed with 2 cuts (Fig. 3, I), except for the TE treatment that showed a fast rise on disease severity in mid-October. However, in the final evaluation, no lesions associated with the pathogen were found, regardless of the treatment.

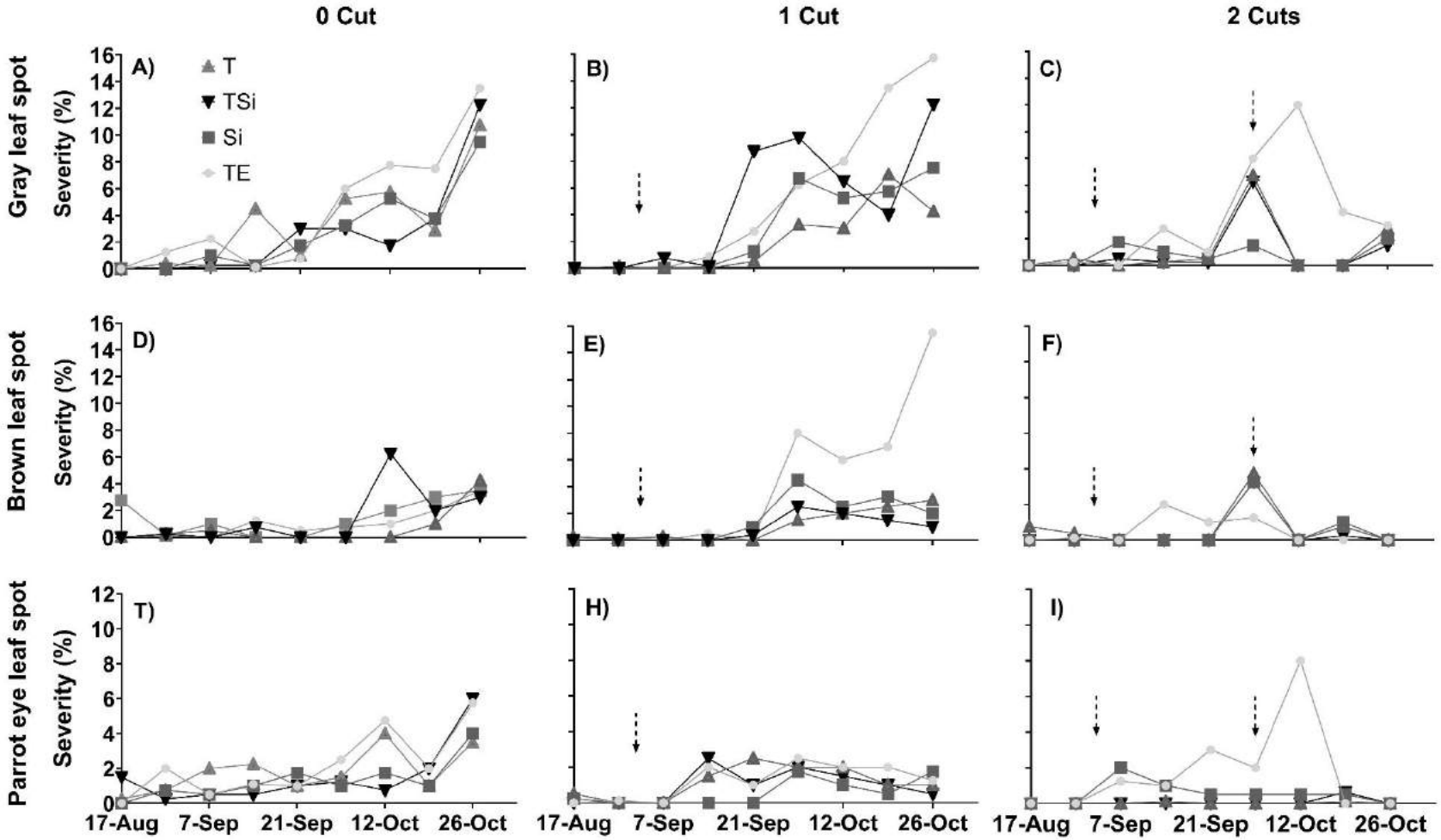

Fig. 3. Severity of leaf spot diseases (season 2018) on Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio, under different soil management, seeds inoculation, and cutoff frequency. TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si = soil modified with 7.8 ton ha^{-1} of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with $6x10^8$ spores mL^{-1} of *Trichoderma atroviride* (TR10), and TSi = soil modified with 7.8 ton ha^{-1} of calcium silicate and seeds pretreated with $6x10^8$ spores mL^{-1} of *T. atroviride*. Black arrow indicates the time of cut.

3.1.3 Area under disease progress curve (AUDPC)

Concerning the AUDPC of the diseases, it varied with the treatment of the soil, seeds inoculation or coating, and cutting (Fig. 4). The ANOVA of AUDPC for gray leaf spot showed highly significant differences ($P < 0.0001$) for cutoff factor and type of management used, however, the interaction was not significant. The type of management that showed the greatest reduction, around of 50%, in the AUDPC was T and Si (Fig. 4, A). Considering the cutoff factor, the best response was found with 2C, showing AUDPC 60% lower than plants without cuts (Fig. 4, B).

For brown leaf spot, the ANOVA of type of management showed significant differences among treatments ($P = 0.0034$) and number of cuts ($P = 0.0001$), but without interaction of factors. TSi and T obtained the lowest value of AUDPC with reduction of 56 and 60%, compared to TE (Fig. 4, C). In regarding to the cutoff effect, one or two cuts were statistical similar to plants without cut (Fig. 4, D), but the AUDPC from plants with two cuts was 75% lower than plants from one cut.

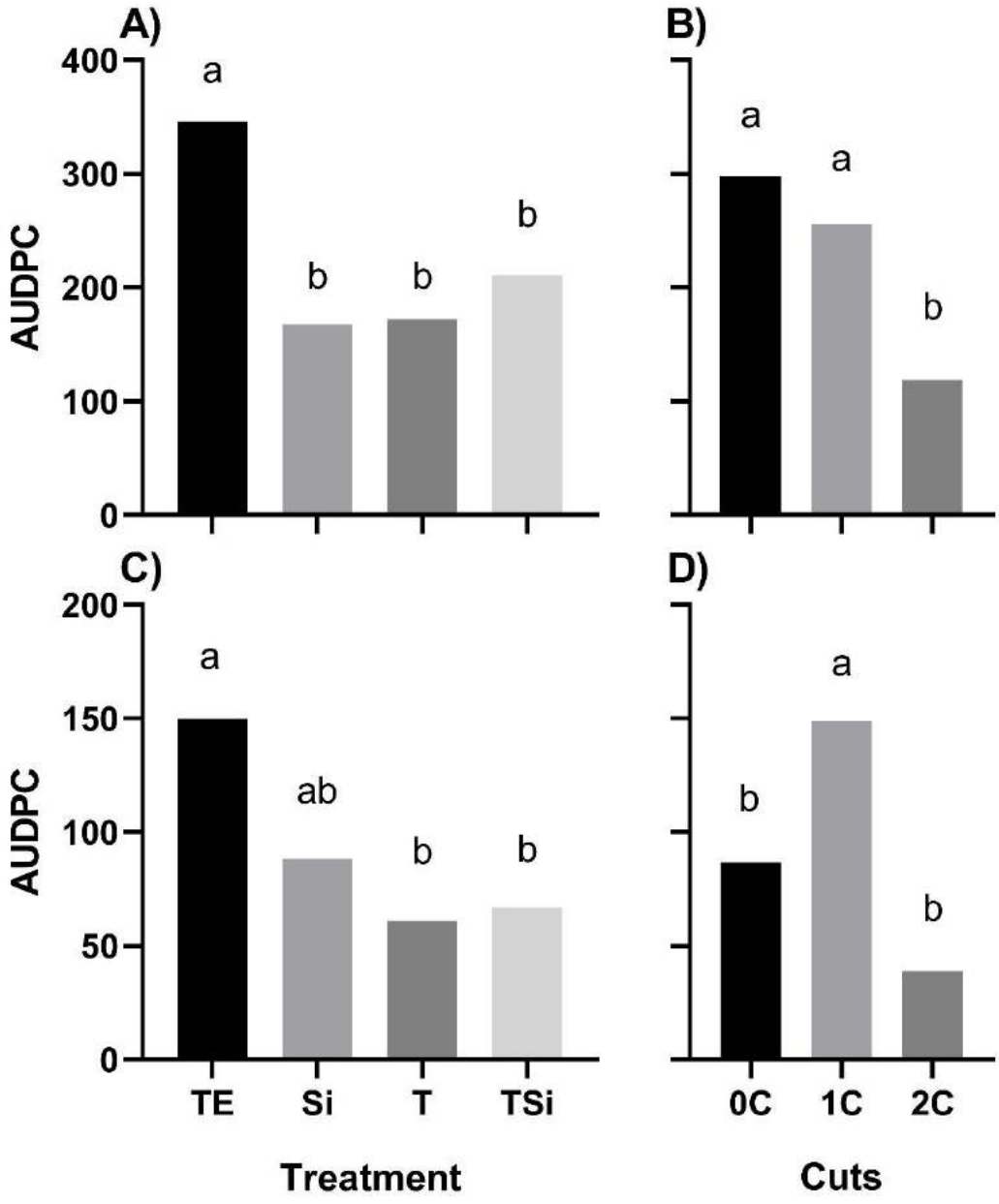

Fig. 4. Area under disease progress curve (AUDPC) of gray leaf spot (A-B) and brown leaf spot (C-D) on Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio (season 2018), grown under different soil management, seeds inoculation, and cutoff frequency. TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si = soil modified with 7.8 ton ha^{-1} of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with 6x108 spores mL^{-1} of *Trichoderma atroviride* (TR10), and TSi = soil modified with 7.8 ton ha^{-1} of calcium silicate and seeds pretreated with $6x10^8$ spores mL^{-1} of *T. atroviride.* 0C=without cutting, 1C=one cut and 2C= two cuts. Bars with different letters indicate statistically significant differences by the Tukey test ($P < 0.05\%$).

3.2 Season 2019

3.2.1 Incidence

The incidence of diseases in 2019 was different of 2018, being recorded only the gray leaf spot infecting *L. multiflorum* plants. The disease incidence reached up to 100% in some treatments and evaluation dates (Fig. 5, A-C). However, the incidence was reduced to 0% during the 15 days after the cut, regardless of the treatment. On most of the evaluation dates, the TE treatment showed a higher incidence than the Si, T and TSi treatments, regardless of the number of cuts. When no cuts was performed (Fig. 5, A), the control treatment obtained 66% of the evaluations with the highest values of incidence, ranging from 55 to 100%, while the remaining treatments (Si, T, and TSi) 11% of the evaluations accumulated the maximum values, which vary in a range between 0-100, 45-100 and 40-100%, respectively. When one cut was applied (Fig. 5, B), TE again accumulated 66% of the maximum incidence values, while Si (16%), T (11%), and TSi (11%) only in sporadic moments. The same tendency was recorded when the plants received 2 cuts (Fig. 5, C), being the TE was the most frequently affected (61% of the total evaluations) in contrast to T, Si, and TSi who obtained the maximum value of the incidence by 16, 11 and 5.5% of the occasions, respectively.

3.2.2 Severity

In the 2019 season, the gray leaf spot was reduced due to cuts made, but mainly by the treatment of soil and/or seeds (Fig. 5, D-F). When the cut was not made (Fig. 5, D), TE reached the highest severity in 100% of evaluations with values up than 10%, while the disease severity was lower than 10% for Si, T and TSi treatments. When one cut was made (Fig. 5, E), the TE obtained the highest value in all evaluations and severity was up than 15%, while Si, T and TSi accumulated a value below than 11, 8 and 15%, respectively. Plants that received two cuts (Fig. 5, F), the gray leaf spot remained higher in TE treatment, up to 19%, meanwhile plants from the Si, T and TSi treatments showed lower severity, showing less than half of the severity observed in the TE in the final evaluation.

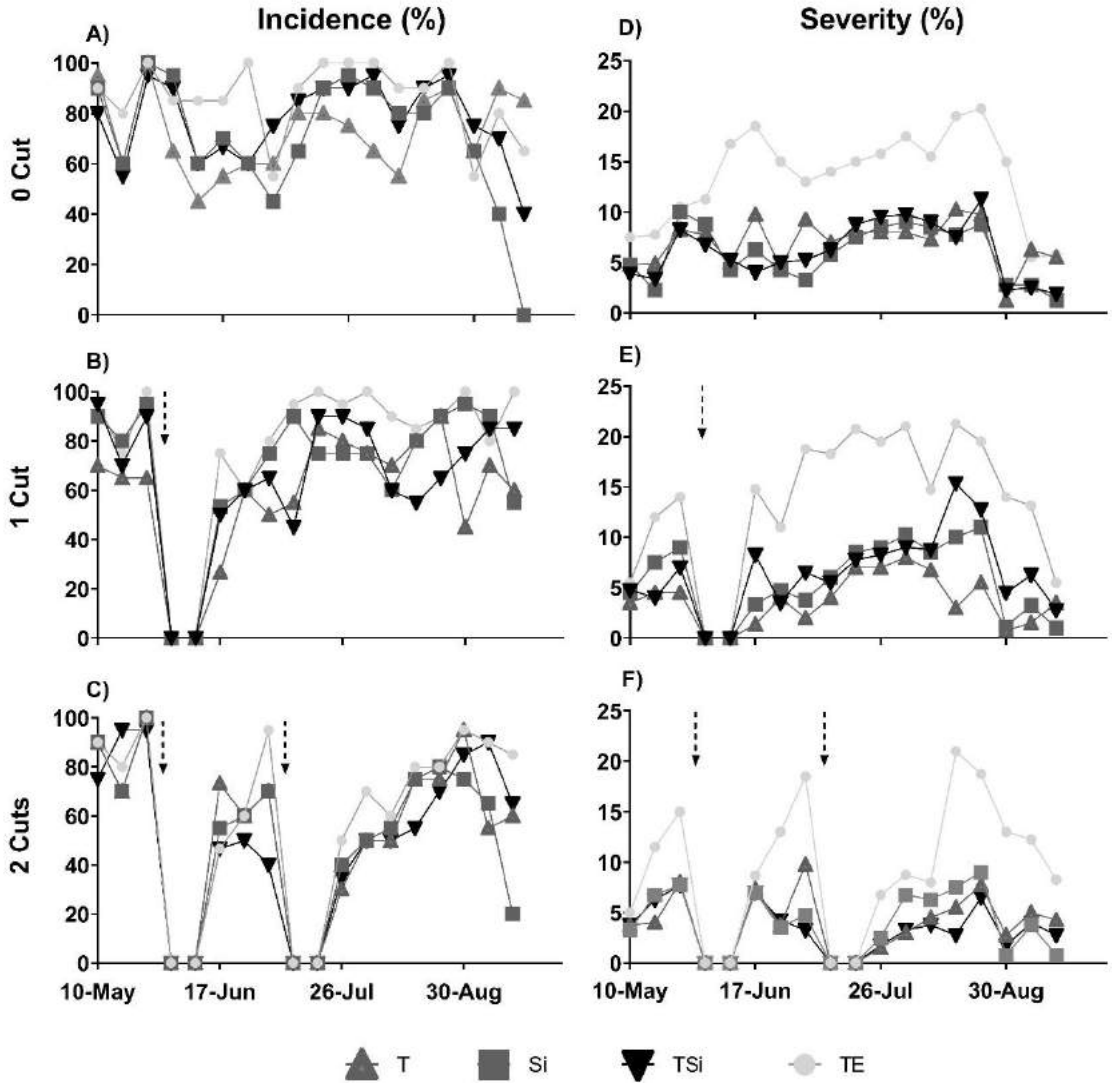

Fig. 5. Gray leaf spot (season 2019) on Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio, under different soil management, seeds inoculation, and cutoff frequency. Incidence= A, B, and C, severity= D, E, and F. TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si = soil modified with 7.8 ton ha^{-1} of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with $6x10^8$ spores mL^{-1} of *Trichoderma atroviride* (TR10), and TSi = soil modified with 7.8 ton ha^{-1} of calcium silicate and seeds pretreated with $6x10^8$ spores mL^{-1} of *T. atroviride*. Black arrow indicates the time of cut.

3.2.3 Area under disease progress curve (AUDPC)

The ANOVA of AUDCP for gray leaf spot showed significant differences (P <0.0001) for management system (TE, T, Si and TSi), for the cut number (0, 1 and 2 cuts), and the interaction between both factors (Fig. 6. A, B, and C, respectively). The AUDPC on plants from T, Si and TSi was reduced significantly, compared to TE, regardless of the number of cuts. Two cuts reduced significantly the AUDPC, regardless of the management system. Furthermore, the AUDPC on plants from the T treatment that received one or two cuts was statistically similar.

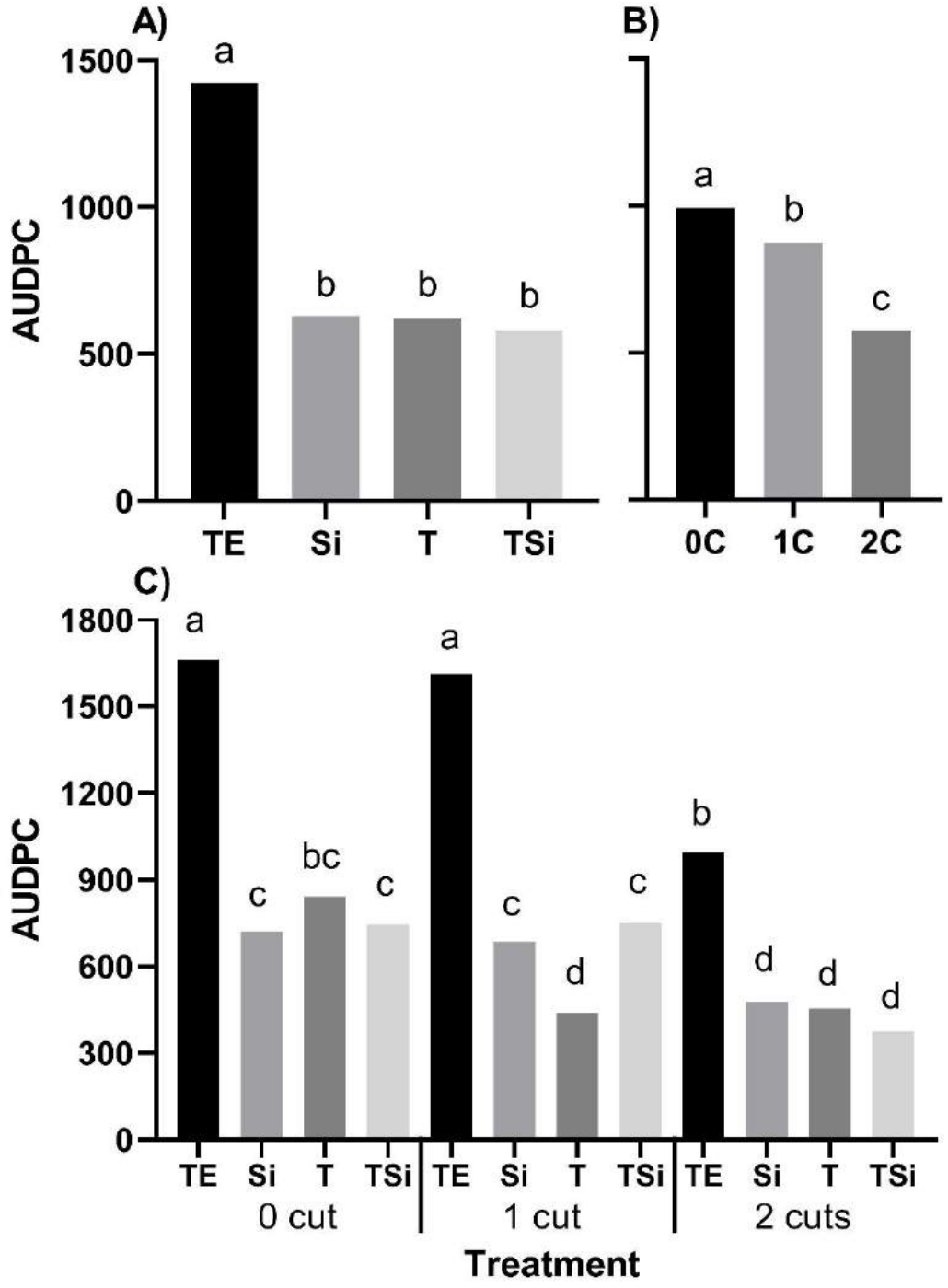

Fig. 6. Area Under Disease Progress Curve (AUDPC) of gray leaf spot on Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio, grown under different management system (MS) and cutoff frequency (CF). TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si = soil modified with 7.8 ton ha^{-1} of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with $6x10^8$ spores mL^{-1} of *Trichoderma atroviride* (TR10), and TSi = soil modified with 7.8 ton ha^{-1} of calcium silicate and seeds pretreated with $6x10^8$ spores mL^{-1} of *T. atroviride*. A) Effect of MS factor, B) Effect of CF factor and C) the interaction of MS and CF factors. Bars with different letters indicate statistically significant differences by the Tukey test ($P < 0.05\%$).

3.3 Determination of silicon concentration in soil and plant tissue

In the 2018 and 2019 seasons, the supply of calcium silicate in the soil increased significantly the Si concentration (Fig. 7, A). However, Si concentration in the soil was not affected by the number of cuts exerted on the plants, regardless of the crop season.

The concentration of silicon in the plant tissue varied according to the soil treatment and the number of cuts made (Fig. 7, B). In both crop seasons, plants without cut and with one cut showed a similar concentration of silicon, however, when the frequency increased to two cuts, the foliar concentration of silicon decreased in all the crop management systems. In the 2018 season, the silicon-free treatments (TE and T) obtained the lowest values in the concentration of the element (from 29 to 36 mg of Si kg^{-1}), compared to Si and TSi, in which were obtained from 39 to 58 mg Si kg^{-1} (an increase of up to 61%). Similar results occurred in 2019, where TE and T obtained from 23 to 27 mg of Si kg^{-1}, while Si and TSi varied from 30 to 39 mg of Si kg^{-1} (up to 44% more than silicon-free treatments). However, in 2019 a slight reduction in the concentration of silicon in plant tissue was observed concerning the year 2018.

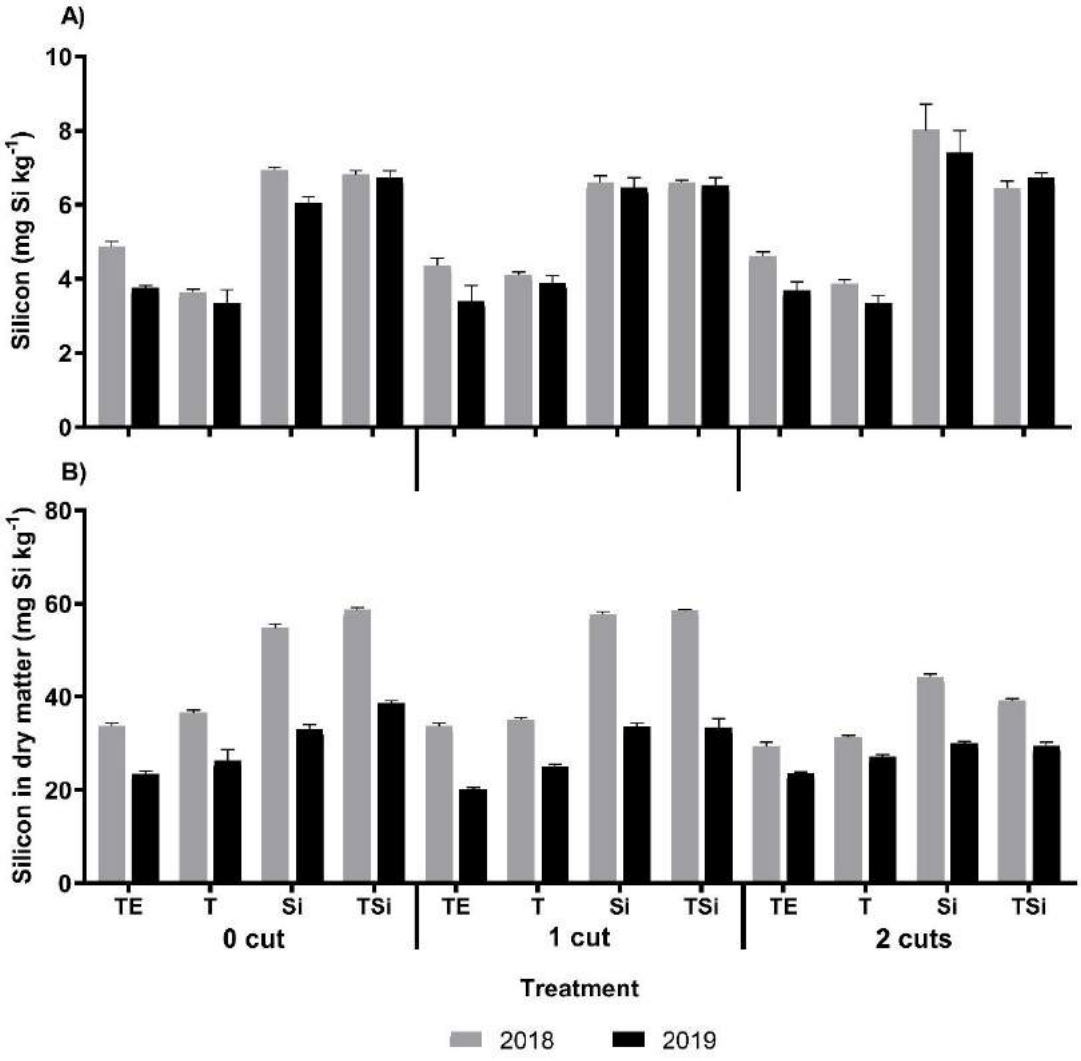

Fig. 7. Silicon concentration in the soil (A) and in the leaf tissue (B) of Italian ryegrass (*Lolium multiflorum* L.) cv. Ponteio, grown under different soil management, seeds inoculation, and different cutoff frequency. TE = Control treatment, plants grown in soil with adjusted pH (6.5) with 7.8 ton ha^{-1} of extra-fine limestone, Si = soil modified with 7.8 ton ha^{-1} of calcium silicate (pH 6.5), T = soil pH adjusted as a treatment TE and seeds pretreated with 6x108 spores mL^{-1} of *Trichoderma atroviride* (TR10), and TSi = soil modified with 7.8 ton ha^{-1} of calcium silicate and seeds pretreated with $6x10^8$ spores mL^{-1} of *T. atroviride.* The bars represent the standard derivation.

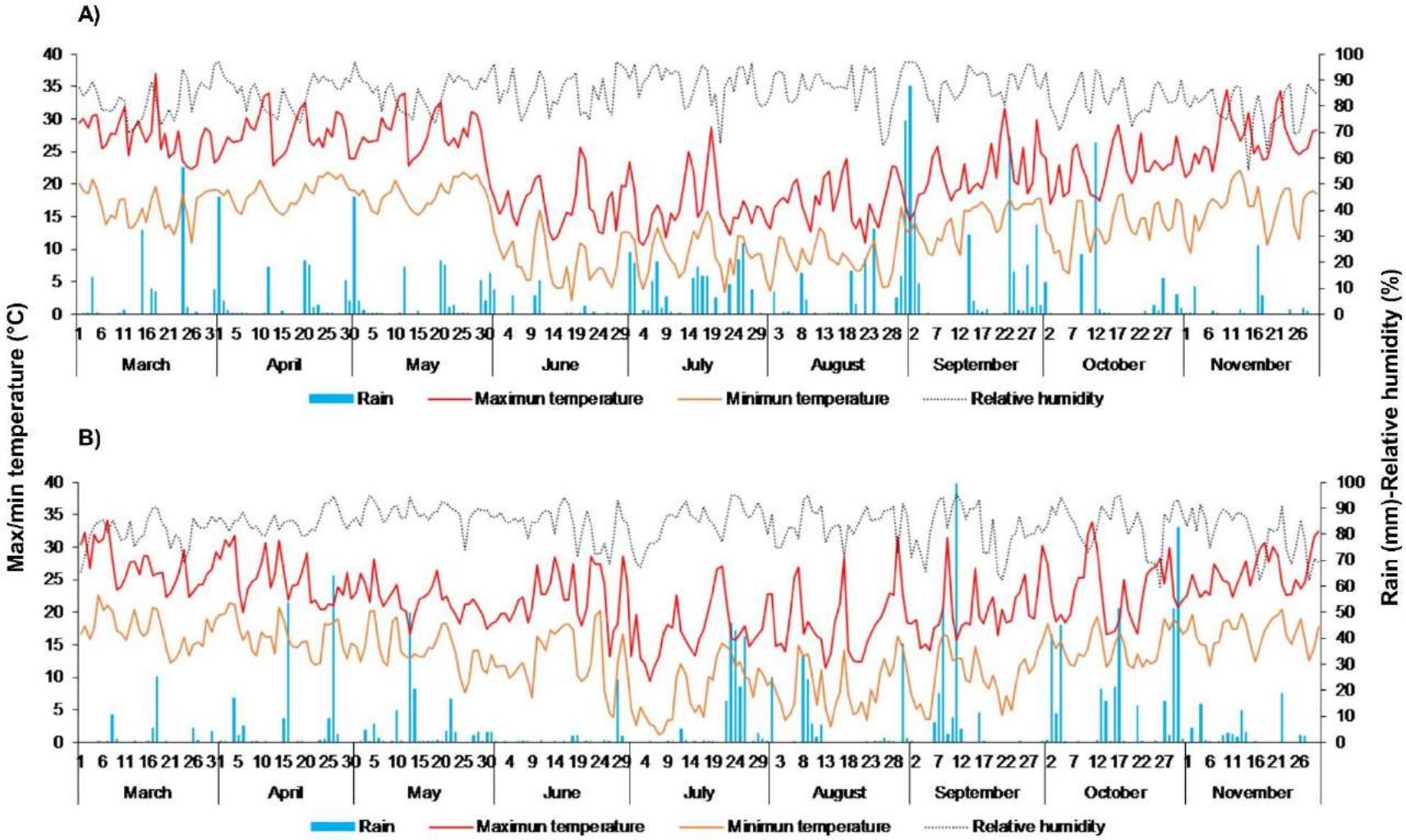

Fig. 8. Meteorological data of the municipality of Pelotas, Rio Grande do Sul, from the period from March to November of the years 2018 (A) and 2019 (B). Source: Agrometeorology-Embrapa laboratory http://agromet.cpact.embrapa.br/

4 Discussion

The results of this study report for the first time the main diseases affecting leaves of ryegrass in Southern Brazil, and the effect of silicon and *Trichoderma atroviride*, alone and associated, on the epidemic development. It was also found that crop protection conferred by these treatments can remain viable for the second growing season, which is adapted to the natural reestablishment model of the pasture, as it is routinely used in this region.

The intensity of the diseases was related to the variations in the environmental factors, such as temperature, relative humidity (RH) and rain (Fig. 8). In 2018, from the period of half August to half October, the temperature and rain increased considerably compared to the second half of July and the first half of August, furthermore, the RH remained from 70 to 96%. The changes in these environmental factors coincided with the sudden increase of incidence and severity of gray leaf spot, brown leaf spot and parrot's eye leaf spot. A similar behavior related to high temperature and RH was observed in 2019 season with the gray leaf spot, because *P. oryzae* began to infect indiscriminately in the period from March to June, the period in which the average temperature varied between 15 to 26 °C and RH between 65 and 94%. A temperature between 26 and 27 °C, and 95% relative humidity favors the infection of *P. oryzae* in rice plants and decreases with a temperature of 22 and 32 °C (Castejón-Muñoz 2008; Singh et al. 2017). However, the progress of gray leaf spot in this study was observed at lower temperatures, in this sense, the sowing date can play a very important role. In 2018, the initial stages of the plants were in low temperatures, given by the sowing date (05/22), however in 2019, the date sowing was not controlled because germination occurred naturally at the end of February (3 months before 2018). The natural regeneration, which takes advantage of the seeds bank of the previous cycle, is a common practice that represents the reality in this and other regions of the Brazil because it reduces the costs of crop establishment. However, the natural regeneration led to the early stages of growth under a regimen of temperature higher to 20 °C and RH higher than 80%, an ideal combination for *P. oryzae* infection. In a study conducted with wheat genotypes (in a region very close to the area of the present study) showed that wheat has been more vulnerable to the attack of *P. oryzae* in terms of incidence, severity and disease index when sown in March compared to May (Coelho et al. 2016). This result suggests that the sowing date is an escape route to reduce the occurrence of gray leaf spot; however, it should be considered that livestock producers working under the crop-livestock integration model, need to take advantage of the end of summer to increase pasture biomass in the shortest possible time.

In the severity of gray leaf spot, in 2019, was possible to detect that the disease increased in greater proportion between the dates from August 16 to 23 in which *L. multiflorum* was at the flowering stage; the same result also happened in the 2018 season. The flowering stage is one of the most important phenological stages because exposure under certain stress conditions (pathogens attack for example) during this period can lead to substantial yield losses in seed-producing plants (Kazan and Lyons 2016). However, in both seasons evaluated, the AUDCP of gray leaf spot was reduced around 50% with the

use of silicon (Si), the inoculation of *T. atroviride* in the seeds (T) or the combination of both treatments (TSi) indistinctly, although with the incidence assessment it was not so clear. Nevertheless, according to Madden et al. (2007), the severity is more important and useful as a measure of the intensity of a disease to estimate yield losses and evaluate the efficiency of management strategies. Furthermore, it was observed that plants with one cut were more prone to infection by *P. oryzae* and *B. sorokiniana*, compared to plants that received two cuts or without cutting. This result can be explained by the fact that plants without cutting were starting the senescence of the fisrt leaves infected, while plants with one cut had a greater amount of green leaves exposed to infection, compared to two cuts treatments, which were still growing; in other words, the two cuts reduced the amount foliar tissue available for infection and at the same time the secondary inoculum.

In addition, the requirement of specific environmental conditions, nutritional status of the plant, or competition for space and nutrients among the pathogens can explain the results of 2018, because the plants from treatments that had less severity of gray leaf spot accumulated greater severity with *B. sorokiniana*. Similar results was reported for the pathosystem of *L. multiflorum*-*Rhynchosporium* spp. and -*Drechslera* spp. (Lam 1983). Authors realized that when diseases occurred together in the same field, the high intensity of *Rhynchosporium* spp. coincided precisely with the low intensity of *Drechslera* spp. For example, in May *Drechslera* spp. was associated saprophytically, only in senescent leaves while in previous months it was associated in all the leaves, in contrast to *Rhynchosporium* spp. that was present in all the leaves in May, in June there was no presence, but *Drechslera* spp. was again present in all the leaves (Lam 1983). This means that the nutrient source available (green or senescente leaves) coupled with environmental conditions at a certain time could favor the type of pathogen established and the level of infection, in this sense, cutting the forage crop can regulate the source of nutrients and also help climate regulation (lowering relative humidity levels, increasing sun exposure and favoring air circulation inside the plant canopy) to create environments less favorable to pathogens.

The use of the cut as a management measure to reduce the source of inoculum, simulating the programmed grazing could help to lowering (even stopping) the development of epidemics within the ryegrass crop, as was shown in both seasons with the reduction of the intensity of the gray leaf spot at the time of the cut. The management of diseases with different cutoff frequency has been little studied, and the present study is the first experimental evidence of the effectiveness of this measure in *L. multiflorum* against the main pathogens causing leaf spots. Cutting or grazing have been suggested in *L. multiflorum* for the management of *Giberella* spp. and *Pyricularia* spp. that attack the spike, since in most of the years the diseases accumulate greater severity at the beginning of the reproductive phase (Nunes and Mittelmann 2009). These activities allows to have a low bearing of the plants and reduce the pressure of the inoculum source, even in areas of seed production (Nunes and Mittelmann 2009).

Furthermore, a positive effect on the disease control was also observed when the seeds were inoculated with *T. atroviride*. The application of this organism was only carried out in 2018, which suggests that

there is a residual effect which remains stable until the 2019 growing season, as observed with the reduction in the intensity of the leaf gray spot disease. This could have happened because the inoculation of the antagonist indirectly facilitated the multiplication in the soil, since in each seed it provided a source of initial inoculum for its establishment. Seed treatment with *Trichoderma* spp. gives protection against concomitant phytopathogens, attached to the seed itself or in the niche where it will be sown (Martínez et al. 2013), however, in this study, the stimulation of defense was also observed. In studies carried out in greenhouse, the stimulation of defense-related enzymes (catalase, peroxidase and phenylalanine ammonia-lyase) was verified when *L. multiflorum* plants were pretreated with the *T. atroviride* TR10 strain, indicating that responses of defense could be systemically induced (see study II). Furthermore, plants grown in soil supplied with silicon showed a similar tendency of plants treated with *T. atroviride* increasing the resistance when challenged with *P. oryzae*. It should be noted that in previous studies in greenhouse, the silicon stimulated the activity of phenylalanine ammonia-lyase in the *P. oryzae* - *L. multiflorum* pathosystem, therefore, the defense response that resulted in disease reduction in field could be related to both physics (cell-wall apposition) and biochemical (secondary metabolites) factors (see chapter II). These results were confirmed in the present research under natural conditions (field) for the gray leaf spot, indicating that any of these strategies could be employed for disease management, especially in the seed-producing areas, where the entry of animals is limited and does not allow the reduction of the inoculum source. In some seed-producing areas, the cut is not made to preserve the best yields, this prevents decreasing the source of inoculum, so the strategies used in this study with silicon and *T. atroviride* can be employed to reduce diseases and indirectly increase the seeds yield.

Regarding the incorporation of silicon in the soil, a beneficial effect was clearly observed, due to disease reduction on plants from silicon-containing treatments. The ability of plants from *Lolium* genus to uptake silicon was previously demonstrated in nine-week-old *L. perenne* plants, which accumulated up to 4% silicon (dry matter) when 10 ton per hectare was incorporated (Nanayakkara et al. 2008). Furthermore, the result obtained with *L. multiflorum* indicated that there is a high relationship between the silicon available in the soil and its concentration in the plant tissue, which has relevant implications, since it confirms that Italian ryegrass is a silicon accumulator plant, and part of the expressed resistance could be explained by the accumulation of this element in the leaf tissue. However, two factors affected the Si concentration in the plant tissue. The first was the number of cuts, which could be due to the duration of the time in which this element is accumulated by the plant, since theoretically plants without cut had more time to carry the silicon towards the leaves than plants with several cuts. And the other factor is the competition for the absorption of silicon, which was higher in 2019 due to the high density of plants in this year generated by the seed bank of the previous year. It should be noted that in 2019 only 1.0 ton per hectare of calcium silicate was reapplied in the same area, which may suggest that

silicon maintains a considerable residual protective effect since it manages to transcend the next growing season as demonstrated by the reduction of the severity of the gray leaf spot.

In previous studies, it was shown that Italian raygras plants even during the vegetative phase when grown in soil modified with calcium silicate (7.8 ton ha^{-1}) accumulated this element three times more than plants that were not grown with this element, and this reduced the severity of *P. oryzae* by 50%, resulting in equal protection conferred by *T. atroviride* inoculation on seeds (see study II). However, in this study under field was also demonstrated a residual effect of the calcium silicate fertilization that lasts for two seasons.

5 Conclusions

The present study is the first evidence of the management of diseases on *L. multiflorum* with a sustainable and lasting approach. Our results show for the first time the importance of the main diseases associated with *L. multiflorum* leaf spots, being the gray leaf spot, the brown leaf spot, and the parrot's eye leaf spot (in order of importance) those with the highest occurrence. Furthermore, we show that the gray leaf spot could be considered the most threatening in this pasture, due to the intensity registered in both evaluated seasons and the favorable environmental conditions.

Our findings also evidenced the effectiveness of the application of silicon in the soil, the inoculated seeds with *T. atroviride*, and the cutoff frequency to reduce the intensity of foliar diseases in field conditions and represent the first management proposals for this grass in Brazil.

Furthermore, we demonstrate that the treatments based on silicon and *T. atroviride* maintain a protective residual effect that remains stable in 2 growing seasons, which adapt to the livestock farming production model.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CONCUSIONS

Seven species of plant pathogenic fungi related to *L. multiflorum* had been reported in Brazil. Two represents new-host association *(Exserohilum rostratum* and *Colletotrichum plurivorum),* three are new reports to Brazil *(Bipolaris sorokiniana, Pyricularia oryzae,* and *Pyrenophora nobleae),* and two are new to science, namely *Curvularia* sp. nov., and *Cercospora* sp. nov.

The gray leaf spot (*Pyricularia oryzae*), brown leaf spot (*Bipolaris sorokiniana*), and parrot's eye leaf spot (*Cercospora* sp. nov.) were the main diseases associated with *Lolium multiflorum,* of which the gray leaf spot was determined as the main disease in the southern region.

The application of silicon in the soil, *Trichoderma atroviride* as coated seed treatment, and the programmed cutoff frequency reduces the intensity of the gray leaf spot and the brown leaf spot in *Lolium multiflorum*.

Treatments based on the application of silicon and *Trichoderma atroviride* maintain a protective residual effect that remains stable in two growing seasons, which adapt to the livestock farming production model.

The findings of this study form a fundamental basis for identifying and quantifying ryegrass diseases in the field, and in the future could help breeders and plant pathologist to design strategies for the control and integrated management in crop-livestock production systems.