ARTIGOS

First report of *Rhizoctonia solani* Kühn AG 2-2 LP in *Zoyzia japonica* Steud in Brazil

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ABSTRACT

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The use of cultivated grasses in Brazil has grown by 40% between 2010 and 2015, and the species *Zoysia japonica* Steud, especially the cultivar 'Esmeralda', corresponds to 81% of the grass market in the country. The most important disease affecting zoysia grass, known as large patch, is caused by *Rhizoctonia solani* and occurs in the Brazilian lawns particularly during winter months. The aim of this study was to contribute to the identification and characterization of the anastomosis group of *R. solani* isolates from lesions typical of large patch collected from 'Esmeralda' grass at gardens and golf courses in the states of São Paulo and Bahia, Brazil. All 12 obtained isolates presented dark-brown colonies with aerial mycelial growth, multinucleated hyphae and absence of concentric zonation or sclerotia, and showed their greatest mycelial growth rate at 25°C. In pathogenicity experiments, except three out of *R. solani* isolates, reduced the growth of zoysia grass. Based on the analysis of sequences of the rDNA-ITS region, the isolates clustered with reference isolates of the anastomosis group AG 2-2 LP. Phylogenetic inference showed that the Brazilian isolates are grouped into two clades that shared the same common ancestral with 96% bootstrap. One of the clades includes only Brazilian isolates while the other one also includes American and Japanese *R. solani* isolates AG 2-2 LP. This is the first report and characterization of *R. solani* AG 2-2 LP in zoysiagrass in Brazil.

Keywords: large patch; 'Esmeralda' grass; identification; phylogeny

RESUMO

Picarelli, M.A.S.C.; Patricio, F.R.A.; Harakava, R.; Rivas, E.B.; Colariccio, A. Primeiro relato de Rhizoctonia solani Kühn AG 2-2 LP em *Zoyzia japonica* Steud no Brasil. *Summa Phytopathologica,* v.46, n.4, p.289-298, 2020.

O uso de gramas cultivadas no Brasil cresceu 40% entre 2010 e 2015, sendo que a espécie *Zoysia japonica* Steud, principalmente a cultivar 'Esmeralda', corresponde a 81% do mercado de gramas cultivadas no país. A doença mais importante da grama zoysia, conhecida por "*large patch*", é causada por *Rhizoctonia solani*, ocorrendo nos gramados brasileiros especialmente nos meses de inverno. O objetivo deste trabalho foi contribuir para a identificação e caracterização do grupo de anastomose de isolados de *R. solani* provenientes de lesões típicas de "*large patch*" coletados em grama esmeralda de jardins e campos de golfe nos estados de São Paulo e Bahia, Brasil. Os 12 isolados obtidos apresentaram colônias marrom escuro, com crescimento micelial aéreo, hifas multinucleadas, e ausência de zonação concêntrica ou escleródios, bem como exibiram sua maior taxa de crescimento micelial a 25°C. Em experimentos de patogenicidade, os isolados de *R. solani*, com exceção de três, reduziram o crescimento das plantas de grama zoysia. Baseado na análise das sequencias da região ITS do rDNA, os isolados agruparam com isolados referências do grupo de anastomose AG 2-2 LP. Inferências filogenéticas mostraram que os isolados brasileiros estão agrupados em dois clados que compartilham o mesmo ancestral comum com 96% boostrap. Em um dos clados estão apenas isolados brasileiros e no outro estão também isolados de *R. solani* AG 2-2 LP americanos e japoneses. Este é o primeiro relato e caracterização de *R. solani* AG 2-2 LP em grama zoysia no Brasil.

Palavras-chave: *"large patch"*; grama Esmeralda; identificação; filogenia

The use of cultivated grasses, especially the species *Zoysia*, *Axonopus* and *Cynodon*, has annually grown in Brazil, although a large portion of the turfgrass market in the country is still based on cropping the native grass *Paspalum notatum* (3). In 2015, cultivated grass sod production reached 24,000 hectares (2), which is an increase of 40% between 2010 and 2015 (32). In contrast to the native grass cultivation, high technology and sustainable practices are employed in the production of cultivated grasses. Zoysiagrass, *Zoysia japonica* Steud, especially the cultivar 'Esmeralda', registration number: 06002-RNC 2018 (6), comprises 81% of the cultivated grasses in Brazil (32), due to its tolerance to different climates, adaptability to a wide variety of soils, and resistance to drought, weeds and wear, as well as low nutrition requirements and slow growth rate. Therefore, low investment is necessary to maintain high quality zoysiagrass lawns. This vegetative propagated grass is suitable for diverse uses, such as residential lawns, large parks with huge landscape areas, airport runways and roadsides. Currently, the major consumers of *Z. japonica* in Brazil are companies responsible for road maintenance (11, 32) on account of the great extent of lawns.

Turfgrass plants are subject to numerous biotic and abiotic disturbances, including diseases caused by fungi of the genus *Rhizoctonia*, a cosmopolitan fungus that causes disease in a wide range

of hosts worldwide. *Rhizoctonia cerealis*, *R. zeae*, *R. oryzae* and *R. solani* have been found infecting a large number of grass species and causing different diseases such as yellow patch, leaf and sheath spot, brown patch and large patch (30).

Rhizoctonia solani Kühn is "a species complex comprising many related but genetically isolated sub-specific groups" (23), called anastomosis groups (AG). *Rhizoctonia solani* has 14 anastomosis groups which are further divided into subgroups. *R. solani* can cause different diseases to turfgrasses, showing variable symptomatology according to the anastomosis group, the turfgrass species, the environmental conditions, and the practices employed in turf management (1). *R. solani* AG 2-2 IIIB has been isolated from cool-season turfgrasses (17), causing brown patch disease, whereas the most common and severe disease of zoysiagrass, large patch, formerly known as zoysia patch (30), is caused by *R. solani* anastomosis group AG 2-2 LP (1, 17, 23), which can also cause disease to other warm-season grasses (22), such as *Eremochloa ophiuroides*, *Stenotaphrum secundatum* (16), *Cynodon dactylon* (1) and *Paspalum vaginatum* (8), all of which are classified as C4 grasses (30). The subgroups of AG 2-2, AG 2-2 IIIB, AG 2-2 IV and AG 2-2 LP, differ in colony morphology, mycelial growth at different temperatures, pathogenicity to different plants (1, 17), and sequences of their ITS regions (10).

Large patch occurs when the weather becomes relatively cool and humid, conditions that prevail especially in the south and southeast regions of Brazil where the seasons are well defined, during the autumn and winter, comprising a continuous period from May to September. The most characteristic symptoms are light brown patches with orange edges, while zoysiagrass plants can have leaves with sheaths that are easily pulled. The patches increase under favorable environmental conditions, reaching large sizes, encroached by weeds. Sometimes, as the patch increases, the grass can recover in the center of the patch. The disease can serious and repeatedly damage lawns, year after year, although the grass can recover as the summer arrives (24).

The disease is difficult to control once it appears in lawns, and several practices can be used for the management of large patch, such as proper fertilization, drainage, and thatch removal, as well as maintenance using the adequate mowing height. Because zoysiagrass is very susceptible, fungicides are also employed to reduce the damage caused by this disease (20). However, only Chlorothalonil is registered in Brazil for use in grass crops (5), although it is not registered for public areas or home gardens.

Considering that large patch, caused by *R. solani*, is becoming increasingly more important in Brazil, the aims of this study were to identify isolates of *R. solani* from large patches of zoysiagrass in turf lawns, characterize the colony morphology and evaluate its growth at different temperatures and its pathogenicity to zoyziagrass, as well as to identify the anastomosis group of isolates and study the phylogenetic relationships between them.

MATERIALS AND METHODS

Culture collection and long-term storage of *Rhizoctonia* isolates

Out of the 24 samples 23 were collected from diseased patches of zoysiagrass lawns in home gardens, golf courses or commercial sod farms in the municipalities of Bragança Paulista, Carapicuiba, Cotia, Ilhabela, Itapetininga and São Paulo, state of São Paulo, Brazil, in the winters of 2013 and 2014. One sample was collected in the early spring of 2016 from a hotel garden in the municipality of Santa Cruz Cabrália, Trancoso, state of Bahia, Brazil. A golf course cup cutter of

10cm diameter and 8cm depth was used to collect the samples from the edges of diseased patches, where, according to Aoyagi et al. (1), the fungus is more active.

To obtain the isolates, segments of the sheath tissue (1cm long), cut from diseased leaves of the samples, were washed under running tap water to remove debris, soaked in 70% ethanol for 30 seconds and subsequently soaked for 3 min in a 0.5% sodium hypochlorite solution, before being rinsed in sterile distilled water and dried on sterile filter paper (23). The segments were placed in Petri dishes containing ¼ diluted potato dextrose agar medium (¼ PDA) added of streptomycin (10 µg/mL). After 24h to 48h incubation in the dark at 25°C, the plates were evaluated under a stereomicroscope (Wild Photomakroskop M400) and an optical microscope (Leiz Laborlux 12) to search for characteristic *R. solani* hyphae; the hyphal tips were successively transferred to Petri dishes containing the same diluted ¼ PDA until pure cultures were obtained. The isolates were stored in dry filter paper strips, which were stored in an ultra-low freezer (14) or in dried wheat kernels (23) before being deposited in the culture collection "Micoteca Mario Barreto Figueiredo" (MMBF), accredited by the World Federation for Culture Collection (WFCC – registration WDCM 942). One additional *R. solani* AG 2-2 IV isolate from MMBF (RH21) was included in the study as a reference. This isolate was collected in Japan, in 1960, and transferred in 2011 to MMBF of "Instituto Biológico", São Paulo, Brazil. A sector of isolate IBRS07 produced the isolate known as IBRS07S. The isolate obtained from the sample collected in Bahia State (IBRS24) was only used for molecular and phylogenetic analyses.

Phenotypic characteristics

The isolates were grown for two weeks on ¼ PDA, at 25°C and 12 h photoperiod, and evaluated for presence or absence of sclerotia, zonation formation, growth form and color of mycelia, based on the scale light brown, medium brown and dark brown.

To assess the number of nuclei, mycelia were evaluated according to Bandoni (4), with some modifications. Mycelium plugs of 5mm diameter were taken from the margins of three-day-old cultures and placed on sterilized microscopic slides, coated with a thin layer of water agar medium, and incubated at 25°C in the dark for 24 h. The developed mycelia were stained with Safranin O and examined under a microscope at 400x magnification. For each isolate, at least ten cells from random hyphae were examined, which were classified as binucleate or multinucleate. This experiment was conducted in duplicate.

Mycelial growth at different temperatures was evaluated based on Aoyagi et al. (1), with some modifications. Mycelial plugs, 10mm diameter, were removed from the margins of three-day-old cultures grown in ¼ PDA and placed on the center of 9cm-diameter Petri dishes containing ¼ PDA. The cultures were incubated in the dark at 10, 15, 20, 25, 30 and 35°C, and 12 h photoperiod. The diameters of colonies were obtained from two perpendicular measurements after every 24h incubation until at least one isolate reached the edge of the Petri dish. The experiment was carried out with four replicates for each isolate and temperature, and each replicate was represented by one Petri dish.

Pathogenicity of *R. solani* isolates on zoysiagrass

Pots containing substrate (13.5cm diameter and 10cm depth) were planted with circular sods of healthy zoysiagrass. Once the grasses were established in the pots, the plants were inoculated with each *R. solani* isolate, according Hyakumachi et al. (17). The inoculum, represented by colonized wheat kernels, was prepared according to Obasa (23). Fifteen wheat kernels colonized (for approximately 10 days) with each isolate were distributed over the grasses on each pot surface. The

pots were watered until field capacity, enclosed in polyethylene bags to maintain high humidity, and transferred to incubation chambers, at 18°C and 12h photoperiod, where they remained for three weeks. Pots from a negative control that did not receive any inoculum were also maintained under the same conditions.

At the end of the experiment, the healthy tissue of treatments was evaluated with digital image analysis. A Canon Power Shot SX40HS was used to photograph 50 cm above the superior surface of each pot. The images were analyzed with the raster graphics editor Corel Photo-Paint™ (12). The green color was inferred as healthy tissue, and the percentage of green pixels was recorded inside a constant rectangle of 1808092 pixels inscribed on each pot surface image. The obtained data were converted into percentage of healthy tissue area, considering the pot surface to be 143.14 cm² (Figure 1).

Two similar experiments were carried out in 2015 and 2016, in a completely randomized design, with five replicates for each isolate, and each replicate was represented by a pot. Results were subjected to analysis of variance (ANOVA) with the software SASM-Agri (9), and means were compared according to Scott-Knott test (P= 0.05). All isolates were re-isolated after the pathogenicity experiment and showed the same characteristics.

Molecular identification of *R. solani* anastomosis group

The isolates had their DNA extracted from mycelia grown in ¼ PDA at 25°C and 12h photoperiod for two weeks, according to the protocol described by Dellaporta et al. (13).

The pair of primers P22-LP, specific for identification of AG 2-2 LP isolates, was used for PCR under the conditions described by Carling et al. (10). Additionally, the primers ITS1 and ITS4 were employed to amplify the internal transcribed spacer regions of rDNA, as described by White et al. (31), with GoTaq G2 Green Master Mix (Promega Corporation, Madison, WI), according to the manufacturer's

protocol. PCR products were analyzed by electrophoresis on 0.8% agarose gel. Amplicons were purified with Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI), following the manufacturer's instructions, and sequenced in both directions using an Applied Biosystems® 3500 Genetic Analyzer. PCR products from the IBRS22 isolate were cloned into the pGEM-T plasmid (Promega Corporation, Madison, WI) as indicated by the supplier, and three DNA clones were sequenced.

Sequence analyses and phylogeny

Consensus sequences for all forward and reverse sequences of each *Rhizoctonia* isolate were obtained with BioEdit version 7.2.6.1 (15) and submitted to the BLAST+ 2.4.0 Program (http://www.ncbi. nlm.nih.gov/blast/Blast.cgi) to search for homologous sequences in GenBank. Multiple sequence alignments involving 13 Brazilian ITS1-ITS4 *Rhizoctonia* sequences and 42 homologous regions of *Rhizoctonia* anastomosis groups from GenBank were first performed by Muscle at MEGA 7 (19) and manually adjusted. The data set underwent pairwise analysis and phylogenetic analyses with two approaches: Neighbor-Joining (NJ) and Maximum Parsimony (MP) in MEGA version 7.0.26 (19).

RESULTS

Rhizoctonia isolates

The 24 collected zoysiagrass samples produced only 12 isolates of *R. solani*: eight from gardens, three from golf courses, and one from a grass sod. In the winter 2013, four isolates were obtained from 13 collected samples, but in 2014 and in the early spring 2016, a greater number of isolates was obtained from the collected samples.

The isolates of *R. solani* identified in this study (Table 1) were deposited in the MMBF mycology collection.

Figure 1. Corel Photo X8 program interface showing green pixels selected inside a constant rectangle inscribed on the image of zoysiagrass infected with *Rhizoctonia solani* (IBRS11) and photographed 50 cm above the superior surface of the pot.

Table 1. Isolates of *Rhizoctonia solani* obtained from zoysiagrass, sampling location, municipality, year of sampling, accession numbers in the mycology collection "Micoteca Mario Barreto Figueiredo" and accession numbers in GenBank.

| Isolate	Sampling location	Municipality	Year	MMBF accession	GenBank accession number
IBRS04	home garden	São Paulo/SP	2013	MMBF54/17	MG264304
IBRS07	home garden	Cotia/SP	2013	MMBF55/17	–
IBRS07S				–	–
IBRS11	home garden	Ilhabela/SP	2014	MMBF56/17	MG264305
IBRS15	home garden	Cotia/SP	2014	MMBF57/17	MG264306
IBRS16	home garden	Cotia/SP	2014	MMBF58/17	MG264307
IBRS18	golf course	Bragança/SP	2014	MMBF59/17	MG264308
IBRS19	home garden	Cotia/SP	2014	MMBF60/17	MG264309
IBRS20	home garden	Cotia/SP	2014	MMBF61/17	MG264310
IBRS21	grass sod	Itapetininga/SP	2014	MMBF62/17	MG264311
IBRS22	golf course	São Paulo/SP	2013	MMBF63/17	MG264313 clone1
					MG264314 clone2
					MG264315 clone3
IBRS23	golf course	São Paulo/SP	2013	MMBF64/17	MG264312
IBRS24	home garden	Trancoso/BA	2016	MMBF65/17	MG264316
RH21		Ibaraki/Japan	1960	MMBF44/11	–
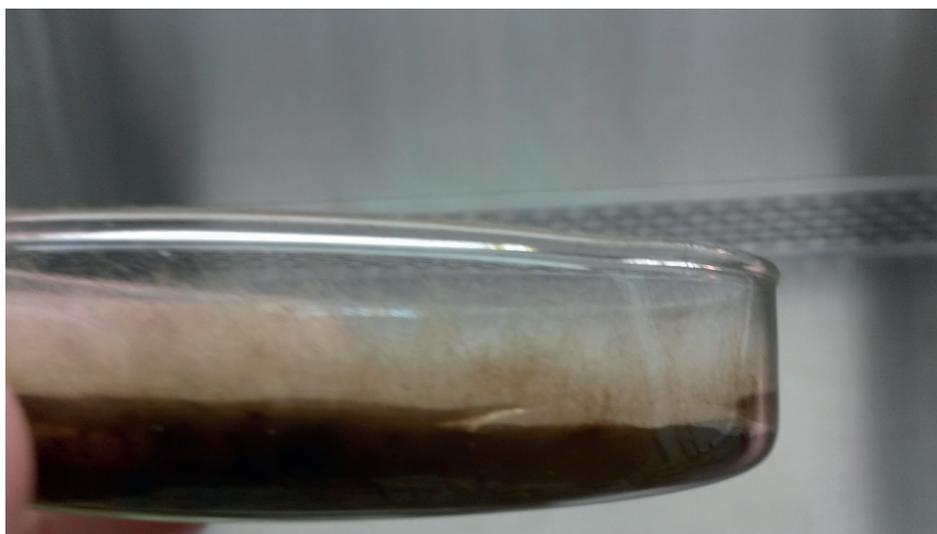
Figure 2. Aerial hyphae of *Rhizoctonia solani* isolate IBRS11.

Phenotypic characteristics

All *R. solani* isolates had multinucleated cells, showed aerial hyphae and no apparent sclerotia, but presented irregular mycelia clusters, as well as diffuse zonation formation, except isolate RH21, which showed distinct sclerotial formation and zonation. The *R. solani* isolates could be statistically clustered into three groups, according to the color of their mycelia: light brown (RH21), medium brown (IBRS15, IBRS19) and dark brown (IBRS04, IBRS07, IBRS07S, IBRS11, IBRS16, IBRS18, IBRS20, IBRS21, IBRS22 and IBRS23).

The isolates showed slow radial growth at 10°C, increasing the growth rate until the maximum of 25°C; their growth rates were between 17.1 (IBRS22) and 26.7 mm/day (IBRS07 or IBRS20). All isolates showed an abrupt decrease in their growth rates at temperatures higher than 30°C, as well as absence of growth at 35°C (Figures 4, 5, and 6).

Pathogenicity of *R. solani* isolates on zoysiagrass

In 2015, the treatments inoculated with the isolates IBRS15, IBRS20 and RH21 did not differ from the negative control. On the other hand, the treatments that received the remaining isolates showed significantly reduced healthy tissue but did not differ from each other (Table 2). In 2016, IBRS11 was the most pathogenic isolate, followed by IBRS07S, IBRS19 and IBRS23, which had zoysiagrass healthy tissue
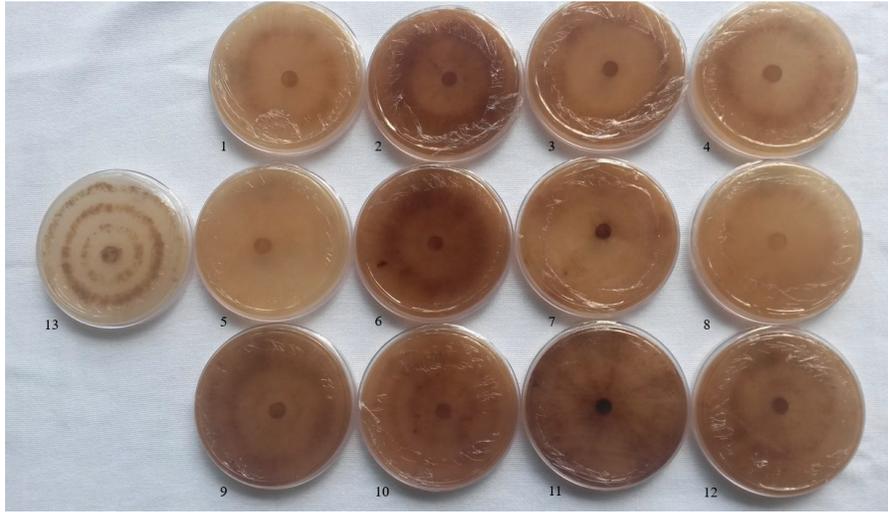
Figure 3. No apparent sclerotia, irregular mycelia clusters, and diffuse zonation formation of *Rhizoctonia solani* isolates IBRS04, IBRS07, IBRS07S, IBRS11, IBRS15, IBRS16, IBRS18, IBRS19, IBRS20, IBRS21, IBRS22 and IBRS23 (1 to 12) in contrast to distinct sclerotial formation and zonation of *Rhizoctonia solani* isolate RH21 (13)
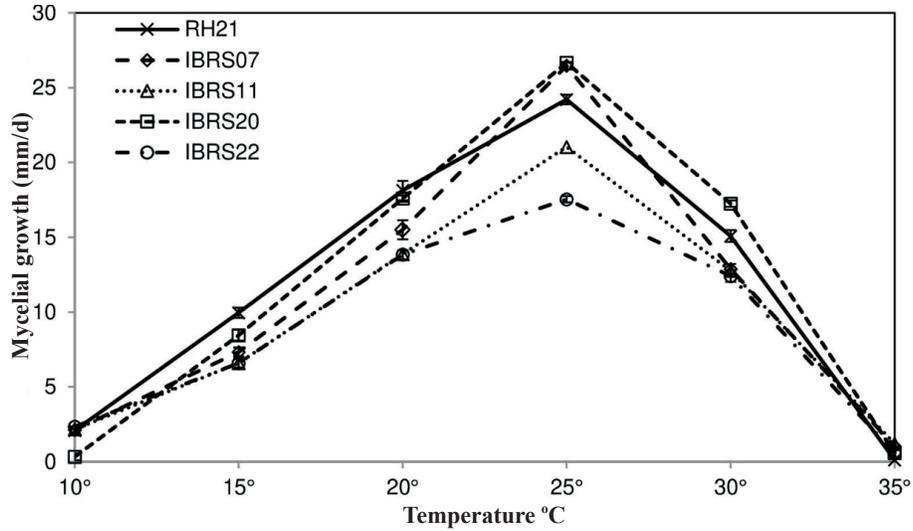

Figure 4. Mycelial growth (mm/day) of *Rhizoctonia solani* isolates (IBRS07, IBRS11, IBRS20, IBRS22) and a *R. solani* AG 2-2 IV isolate (RH21) at 10, 15, 20, 25, 30 and 35°C. Bars show the standard error.
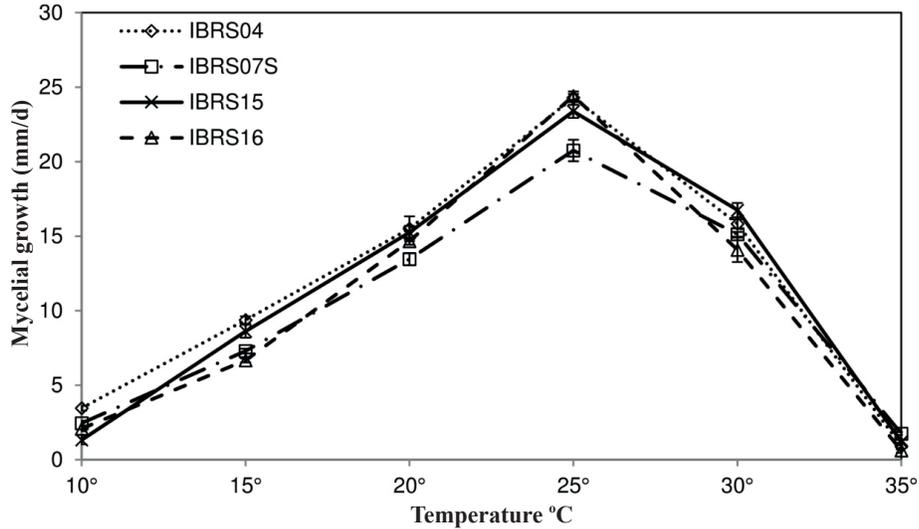

Figure 5. Mycelial growth (mm/day) of *Rhizoctonia solani* isolates (IBRS04, IBRS07S, IBRS15, IBRS16) at 10, 15, 20, 25, 30 and 35°C. Bars show the standard error.
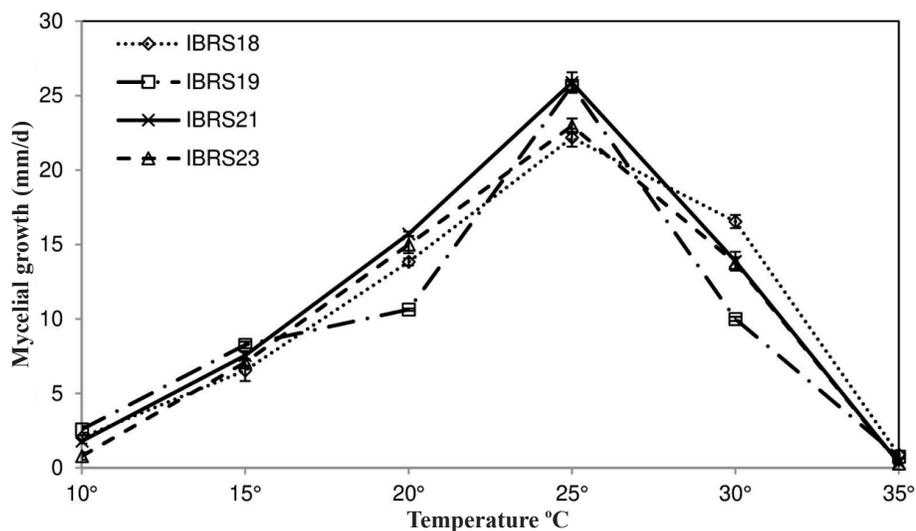
Figure 6. Mycelial growth (mm/day) of *Rhizoctonia solani* isolates (IBRS18, IBRS19, IBRS21, IBRS23) at 10, 15, 20, 25, 30 and 35°C. Bars show the standard error.

Table 2. Green tissue (cm²) of zoysiagrass cultivated in pots after inoculation with isolates of *Rhizoctonia solani,* in experiments carried out in 2015 and 2016.

Treatment	April 2015		May 2016	
	Healthy tissue[y] cm²		Healthy tissue[y] cm²	
Negative control	104.62	a[z]	137.70	a[z]
RH21	101.40	a	133.62	a
IBRS04	69.94	b	122.07	b
IBRS07	76.58	b	121.38	b
IBRS07S	74.75	b	103.27	c
IBRS11	67.24	b	57.21	d
IBRS15	101.07	a	131.00	a
IBRS16	68.91	b	122.37	b
IBRS18	56.73	b	122.60	b
IBRS19	62.98	b	113.62	c
IBRS20	96.95	a	134.60	a
IBRS21	74.95	b	137.62	a
IBRS22	73.25	b	125.38	b
IBRS23	70.01	b	113.91	c
C.V.%	16.99		8.93	

[y]Healthy green tissue, evaluated by the raster graphics editor Corel Photo Paint X8 (Corel Corporation, 2016). Green color was inferred as healthy tissue. The percentage of green pixels was estimated in a rectangle of 1,808,092 pixels, inscribed on the surface image of each pot. Data were converted into percentage of healthy tissue area, considering the pot surface 143.14 cm.

[z]Means followed by the same letter do not differ significantly according to Scott-Knott test (*P*=0.05).

reduced by 56.23, 24.05, 16.82 and 16.61%, respectively. These were followed by the remaining isolates, except RH21, IBRS15, IBRS20 and IBRS21, which did not differ from the negative control (Table 2).

Molecular and phylogenetic analyses

The 12 *R. solani* isolates obtained in Brazil produced a 400bp product when amplified with primers P 22-LP, which are specific for *R. solani* AG 2-2 LP.

Using ITS1/ ITS4, 676bp fragments were obtained; in addition, fragments of the same size obtained from three clones of the isolate IBRS22 were sequenced. The sequences were deposited in GenBank under the accession numbers shown in Table 1.

All 12 Brazilian sequences showed high homology with the isolates of *R. solani* AG 2-2 LP anastomosis group when aligned to homologous regions from *R. solani* sequences in GenBank. Pairwise identity comparisons between the isolates of the present study and those from GenBank exhibited high percentages, ranging from 96.1% to 100% for the *R. solani* AG 2-2 LP group. The sequences of isolates IBRS20, IBRS22 clone 1, had 100% identity with the sequences of three *R. solani* isolates obtained from zoysiagrass from Texas and the USA (FJ746981, FJ746982 and FJ746983), while the same percentage was found for the sequences of IBRS23 and IBRS22 clone 3 of the isolates from Texas (FJ746919 and FJ746920).

The lineages AB054866, AB054867, AB054868 and AB054869 from Japan also presented 100% identity with each other.

Although 25 sequences were used for the alignment and pairwise analysis, 45 accessions of different *R. solani* anastomosis groups were used to infer the phylogenetic tree, i.e., only one representative from a set of sequences with 100% identity. The topologies from trees reconstructed using NJ and MP were very similar (data not shown), and the strong bootstrap supports the Brazilian isolates as belonging to the same clade of *R. solani* AG 2-2 LP lineages, sharing the same common ancestor (Figure 5). The Brazilian lineages of *R. solani* AG 2-2 LP were grouped with lineages of the same anastomosis group from the USA and Japan. However, the Brazilian lineages IBRS11, IBRS15, IBRS16, IBRS19 and IBRS24, from different counties in the states of São Paulo and Bahia, formed a cluster that share the same ancestor with other AG 2-2 LP lineages
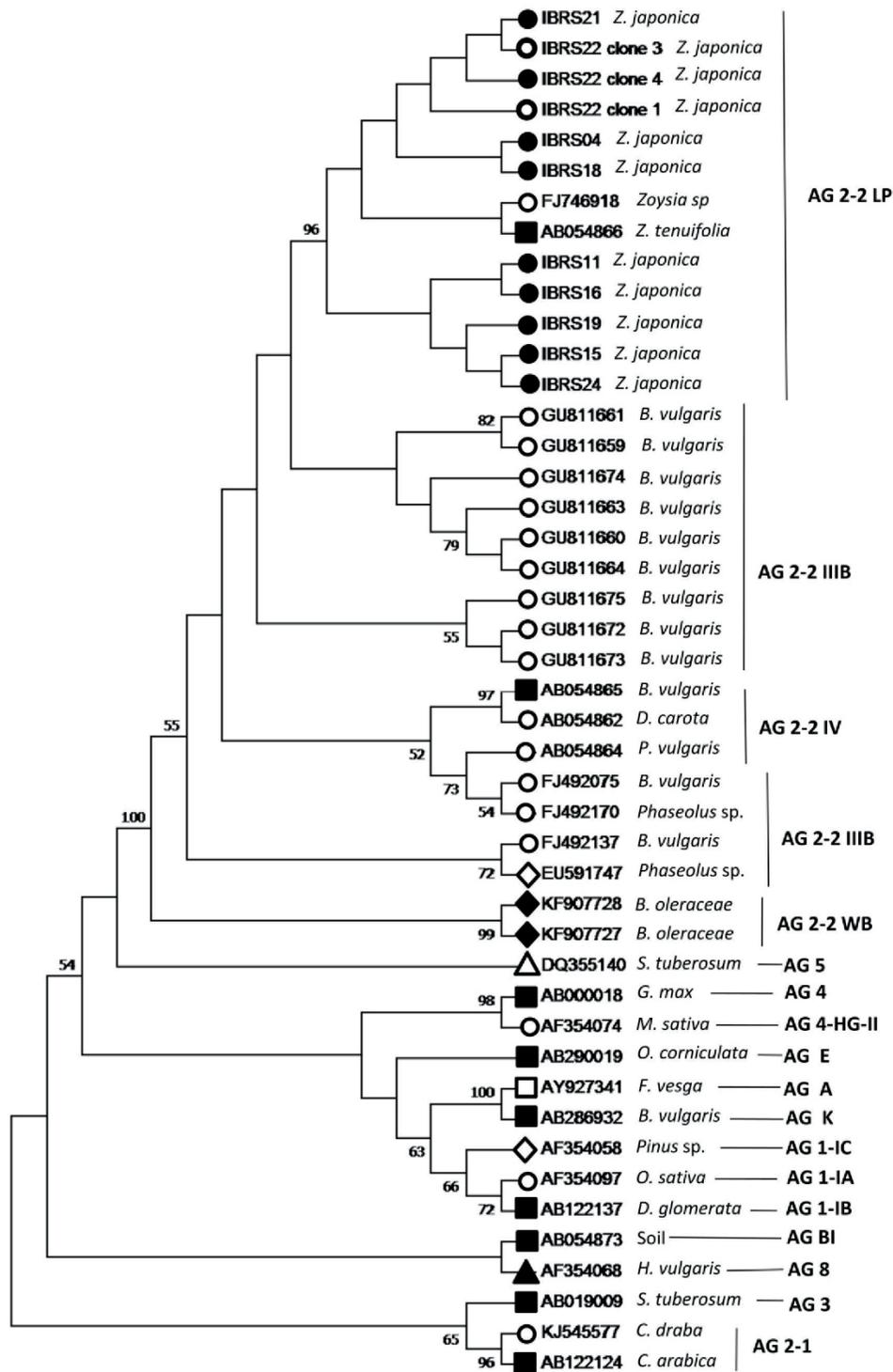
Figure 7. Phylogenetic tree inferred by the Maximum Parsimony method using the Tree-Bisection-Regrafting (TBR) algorithm of the rDNA-ITS region of *Rhizoctonia solani* Kühn. Bootstrap values shown near the branches represent the percentages of 1,000 replicates. Bootstrap values lower than 50 were omitted. The analysis involved 45 nucleotide sequences and a total of 741 positions in the final dataset. The symbols represent the origin of *Rhizoctonia solani* isolates: ●Brazil; ○United States; ◑Brazil and the United States; ◇Canada; ■Japan; ▲Australia; ◆Vietnam; △France; and □Italy

from Brazil, the USA and Japan (Figure 7).

On the other hand, the value of 100 bootstraps provides robustness to cluster AG 2-2 subtypes.

DISCUSSION

Few studies were carried out in Brazil focusing on the causal agent of large patch, although it is considered the main disease affecting zoysiagrass lawns in Brazil (29) and other countries (30).

In the present study, sampling was concentrated in home gardens and sport fields since their management requires high fertilization and frequent irrigation, while the density and increasing poorly drained soils have favored infection by *Rhizoctonia* spp. (30), in contrast to the practices employed for huge lawns.

Learning the specific symptoms of the disease is important for correct grass sampling, avoiding areas with other diseases, pests or physiological disorders. Different types of patches could/can be observed in zoysiagrass during the winter in Brazil. In the second year of study, the authors were able to more clearly identify the large patch lesions, especially those from which sheaths could be easily pulled.

The best method to isolate *R. solani* used the sheaths of zoysiagrass leaves; the same method was adopted by Aoyagi (1), Hyakumachi et al. (17), Obasa (23), Obasa et al. (24) and Obasa et al. (25). Other attempts to isolate the fungus from thatch or using bean baits were not effective because bacteria and nematodes that live freely in the rhizosphere soil (data not shown) frequently contaminated the culture plates. Similarly, 61% of the 1,706 isolates, in a 6-year study carried out by Aoyagi et al. (1), were obtained from plant tissue, although the authors were also able to isolate the pathogen from thatch (16.9%) and flax baits (22.1%).

All *R. solani* isolates of this study showed phenotypic characteristics of AG 2-2 LP anastomosis group, described by Aoyagi et al. (1), Hyakumachi et al. (17) and Obasa et al. (23). The mycelia had multinucleated cells and exhibited aerial hyphae that were initially whitish, becoming dark brown, with irregular clusters of mycelia and diffuse zonation, after growing for two weeks at 25°C. They clearly differed from the evident zonation and sclerotium formation of the RH21 lineage, which is characteristic of the anastomosis group AG2-2 IV (1).

The mycelial growth of the present isolates was optimal at 25°C, which was similar to that observed by Hyakumachi et al. (17) and Obasa (23) for isolates of *R. solani* AG 2-2 LP, whereas Aoyagi et al. (1) found optimal growth of *R. solani* AG 2-2 LP at 23°C. The Brazilian isolates showed a very low growth at 10°C and no growth at 35°C, similar to the mycelial growth observed by Hyakumachi et al. (17), which was very low at 5°C or 35°C. Daily growth appears to vary among AG 2-2 LP isolates. The Brazilian isolates showed maximum growth of 17–27 mm/day; the same result was observed by Obasa (23) for 58% isolates from Kansas and Missouri (EUA), where values were higher than those of nearly 15mm/day observed by Aoyagi et al. (1) and Hyakumachi et al. (17) in Japan. However, differences between isolates of *R. solani* from the same anastomosis group are expected. It must be considered that the isolates studied by Aoyagi et al. (1) and Hyakumachi et al. (17) were obtained from other *Zoysia* species: *Zoysia matrella* (1) and *Zoysia tenuifolia* (17). On the other hand, in the present study and in that carried out by Obasa (23), isolates were obtained from *Zoysia japonica*, namely from the cultivar 'Meyer', the most popular zoysiagrass cultivar in the transitional zone of the USA (27), and from the cultivar 'Esmeralda', which is becoming very popular in Brazil (2).

Most *R. solani* isolates of the present study were pathogenic to zoysiagrass, except IBRS15, IBRS20 and IBRS21. Although they have been isolated from symptomatic diseased patches, these isolates may have lost their pathogenicity due to the successive hyphal-tip transfers under the laboratory conditions. The remaining isolates showed variable levels of virulence on *Z. japonica*, but IBRS11 was more virulent than the remainder of isolates in the second experiment; similar results were observed by Aoyagi et al. (1) and Hyakumachi et al. (17). The *R. solani* AG 2-2 IV isolate RH21 was not pathogenic, which was expected because Aoyagi et al. (1) observed that this anastomosis group was not pathogenic to warm grasses, such as St. Augustine, Bermudagrass and zoysiagrass.

The use of digital image analyses was suitable for evaluating the damage caused by large patch in zoysiagrass, especially because this is a non-subjective criterion, while some methods adopted in previous studies, such as a rating scheme (17) or disease index (1), are more subjective. The digital software used by Obasa (23) was similar but specific for turfgrass analysis, Turf Analysis – SigmaScan Pro Version 5.0 (18), to evaluate large patch severity in zoysiagrass.

The biological characteristics that have helped classify Brazilian isolates as AG 2-2 LP found strong support in molecular and phylogenetic results for the rDNA-ITS region.

Direct sequencing of amplicons of the rDNA-ITS region from the isolates revealed high heterogeneity of sequences, characterized by overlapping peaks in chromatograms at some positions within the sequence, which can be explained because *R. solani* is a multinucleate and heterokaryotic fungus (23), which can have different rDNA units within the same nucleus or in different nuclei (26). Thus, the three clones obtained from the IBRS22 isolate differed in a few nucleotides, showing 99.7 and 99.9% identity. Salazar et al. (28) also observed high heterogeneity for *R. solani* AG 2-2 isolates from leguminous plants. Molecular approaches were used to classify *R. solani* into AG 2 anastomosis group (7, 10, 17, 28) due to the heterogeneity and the difficulty in differentiating sub-groups.

Pairwise analysis showed that Brazilian isolates of *R. solani* exhibited 98.2% to 100% identity with AG 2-2 LP isolates from GenBank. Carling et al. (10) highlighted that AG 2-2 LP isolates have very similar ITS sequence, which is also supported by Li et al. (21); irrespective of the host or geographical origin, the latter author detected little genetic variation among isolates from zoysiagrass, Bermudagrass, centipedegrass and St. Augustinegrass in fingerprinting markers from Amplified Fragment Length Polymorphism DNA.

According to the phylogenetic analyses, the Brazilian isolates clustered in the same sub-group with other AG 2-2 LP isolates sharing the same ancestor of AG 2-2 LP isolates from GenBank, regardless of the host and geographical distances from where they were collected.

The high similarity between the *R. solani* AG 2-2 LP isolates of this study and those of previous studies (10, 17) may be due to the mode of propagation of zoysiagrass, although they have different origins. This type of grass is mostly vegetatively propagated, from sods or sprigs, since the seeds present low germination, high dormancy, and poor seedling vigor, resulting in limited seed offerings (27). On the other hand, *R. solani* AG 2-2 LP is a pathogen that persists in the plants throughout the year, regardless of the period when the symptoms appear. Aoyagi et al. (1) could recover *R. solani* AG 2-2 LP isolates from grasses without apparent symptoms in a golf course affected by large patch throughout the year, although the disease only occurs when environmental conditions are favorable to the pathogen and unfavorable to the grass.

Therefore, the exchange of zoysiagrass plant material between different locations and countries could disseminate the pathogen. *Zoysia* species were introduced in the USA at the beginning of the 20th century, i.e., *Z. japonica* from North Korea and Japan, *Z. matrella* from Japan, the Philippines, Australia, Taiwan and Indonesia, and *Z. tenuifolia* from Japan, through seeds or vegetative propagative material (27). In Brazil, the introduction of *Z. japonica* in the seventies (32) probably occurred the same way. This could explain why the Brazilian isolates IBRS20, two clones of the isolate IBRS22, and IBRS23 presented 100% identity with the American isolates.

Detection of isolate IBRS21 in a sod farm from Itapetininga, São Paulo State, shows that the disease can be spread. This is especially true because Itapetininga reports the highest sod production in Brazil, and the state of São Paulo is responsible for 50% of the national zoysiagrass sod production (2).

This is the first study to characterize *R. solani* isolates obtained from zoysiagrass exhibiting symptoms of large patch in Brazil and to identify it as *R. solani* AG 2-2 LP present in the *Z. japonica* lawns in different regions in Brazil.

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