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SHORT COMMUNICATION

rDNA-based characterization of a new binucleate *Rhizoctonia* spp. causing root rot on kale in Brazil

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Abstract In this paper we present the first report of the occurrence of a binucleate *Rhizoctonia* spp. causing hypocotyl and root rot in kale in Brazil. *Rhizoctonia* spp. were isolated from kale (*Brassica oleracea* var. *acephala*) with symptoms of hypocotyl and root rot. The isolates, characterized as binucleate *Rhizoctonia* spp., did not show an anastomosis reaction with any of the binucleate *Rhizoctonia* spp. testers used. The pathogenicity of the isolates was tested under greenhouse conditions; all isolates were pathogenic and showed different symptom severities on kale. The ITS-5.8S rDNA sequences of kale isolates and 50 testers (25 binucleate *Rhizoctonia* spp. and 25 *Rhizoctonia solani*) were compared in order to characterize the genetic identity of *Rhizoctonia* spp. infecting kale. The kale isolates showed genetic identities ranging from 99.3 to 99.8% and were phylogenetically closely related to CAG 7 (AF354084), with identities of 98.5 and 98.7%. It is suggested that the binucleate *Rhizoctonia* spp. causing hypocotyl and root rot on kale Brazil comprises a new AG not yet described.

Keywords Anastomosis group · ITS1 · ITS2 · 5.8S rDNA

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Rhizoctonia species are basidiomycete fungi associated with important agricultural and horticultural crops grown all over the world. These fungi are able to colonize and/or infect seeds, roots, leaves, stems and fruits of many crops. *Rhizoctonia* spp. comprise an important multinucleate species complex represented by *R. solani* and several others which are either mononucleates or binucleates. Up to now, there are 14 anastomosis groups (AG) of *R. solani* described: AG-1 to AG-13 and AG-BI (Carling 2000). Binucleate *Rhizoctonia* species are grouped either in seven AGs (CAG1 to CAG7) in the USA (Burpee et al. 1980) or 19 AGs (AGA to AGS) around the world (Ogoshi 1987).

While most of the *R. solani* AGs are recognized as important plant pathogens, the binucleate *Rhizoctonia* species are mostly considered as mycorrhizal or having a role as biocontrol agents (Burpee and Goulty 1984; Cardoso and Echandi 1987a, b; Herr 1988; Escanbde and Echandi 1991; Harris and Adkins 1999). Thus, only a few of the known binucleate *Rhizoctonia* spp. AG are pathogenic. Non-pathogenic binucleate *Rhizoctonia* spp. are commonly found in soil organic matter or in plant debris. In a few Brazilian fields sampled, they were mostly associated with beans, peanuts (Ceresini and Souza 1997) and

soybeans (Fenille et al. 2002) primarily infected by *R. solani* AG 4 HG-I or AG 2.2 IIIB, causing damping-off and root rot symptoms. Most of these binucleate *Rhizoctonia* spp. were non-pathogenic and were considered to have potential for biocontrol (Harris 2000). Pathogenic binucleate *Rhizoctonia* spp. isolates belonging to AG E have been observed associated with soybean in the USA (Ploetz et al. 1985) and Indonesia (Naito et al. 1993). A few other binucleate *Rhizoctonia* spp. isolates were pathogenic to potato roots (Carling and Leiner 1990) and yacon (Fenille et al. 2005). Distinct AGs of *R. solani* have already been identified causing disease on different vegetable crops in Brazil (Bolkan and Ribeiro 1985; Kuramae et al. 2003). However, there are no reports on the occurrence of pathogenic binucleate *Rhizoctonia* causing disease on vegetable crops in Brazil. Particularly on kale, a *Rhizoctonia*-like disease has been observed causing outbreaks in São Paulo State vegetable-growing areas. Hypotcotyl and root rot symptoms have been observed on kale.

Considering the local importance of this *Rhizoctonia*-like disease for kale production, the objective of this study was to characterize the *Rhizoctonia* spp. isolates infecting kale by determining their nuclear condition, hyphal anastomosis grouping, virulence and genetic identity with other *Rhizoctonia* spp. based on sequencing analysis of the ITS-5.8S rDNA region. Our hypothesis was that a binucleate *Rhizoctonia* spp. is the primary agent of a hypotcotyl and root rot disease detected on kale.

The isolates were collected from kale (*Brassica oleracea* var. *acephala*) cultivated in Lençois Paulista, SP, Brazil, with hypocotyl and root rot symptoms. Small sections (0.5–0.7 cm long) were cut from the edge of an advancing lesion on each diseased root sample. These samples were surface-sterilised by immersion in 70% ethanol for 30 s, 2% sodium hypochlorite for 30 s, washed in sterile distilled water (SDW) and placed directly on Ko and Hora medium (1971). The nuclear condition and hyphal anastomosis grouping of the isolates were determined using the procedure of Ceresini et al. (1996). Hyphal cells were examined microscopically at 200 and 400× and nuclei were counted in 20 cells per isolate. Kale binucleate *Rhizoctonia* spp. isolates were paired against the AG testers (AG A, AG Ba, AG Bb, AG Bo, AG C, AG D, AG F, AG G, AG O, AG P and AG Q) available in our laboratory. Hyphal anastomosis was observed at 400× magnification using light microscopy after staining the vegetative cells with a 0.03% safranin-O aqueous solution and a 3% KOH aqueous solution. All isolates of *Rhizoctonia* spp. obtained were binucleate and did not show anastomosis reactions with any of the binucleate *Rhizoctonia* spp. testers used. However, in the absence of a few AG testers of binucleate *Rhizoctonia* spp. being not available in our laboratory (CAG 1, CAG 3, CAG 4, CAG 5, CAG 6, CAG 7, AG L, AG T and AG U), a phylogenetic approach was used for characterizing the unknown kale isolates. We based this approach on the comparative analysis of sequences of the ITS-5.8S rDNA region from all the AGs of binucleate *Rhizoctonia* spp. as well as the *Rhizoctonia solani* available and the unknown kale isolates. The

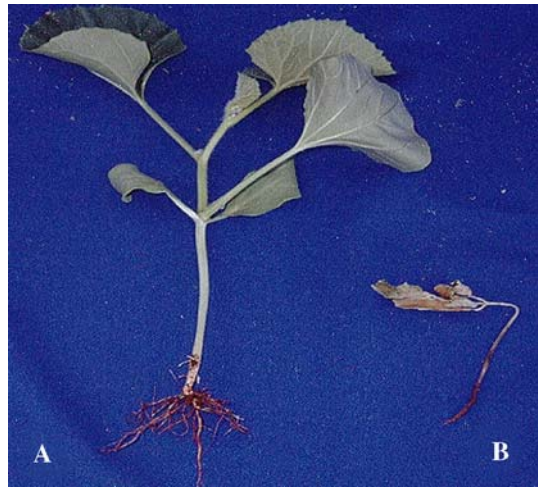

Fig. 1 (**A**) is the control. (**B**) Pathogenicity of isolate CO4 of binucleate *Rhizoctonia* spp. to kale after 20 days of inoculation

Table 1 Virulence of binucleate *Rhizoctonia* spp. on kale 20 days after inoculation

Isolate	Disease severity index[1,2]
CO4	2.00a
CO2	1.92ab
CO1	1.66b
CO3	1.66b
Control	0.00c

[1] Disease severity scored as 0 = no symptoms, 1 = hypocotyl and cotyledon symptoms, 2 = pre-emergence damping-off, 3 = post-emergence damping-off

[2] Means followed by the same letter within a column are not significantly different by the Tukey test ($P = 0.05$)

sequences of the unavailable AGs were obtained from the GenBank. This approach provided us with some information about the placement of the unknown kale isolates among the AGs described for binucleate *Rhizoctonia* spp. worldwide. The use of a molecular tool is not currently accepted as a replacement criterion for the hyphal anastomosis characterization of AGs of *Rhizoctonia* spp. However, in certain instances the sequencing of the ITS-5.8S rDNA region has been used to separate subgroups of *R. solani* when anastomosis itself cannot separate them (Kuninaga et al. 1997).

The pathogenicity of the isolates was tested under greenhouse conditions at 25 ± 2°C using the substrate and inoculum preparation recommended by Fenille and Souza (1999). A completely randomized block design with five replications was used. After transferring the inoculum to the substrate, thirty seeds of kale cv. Portuguesa were sown per pot. Disease severity was assessed, 20 days after inoculation, using the scale described by Chung et al. (1988) and the fungus was re-isolated. All isolates caused hypocotyl and root rot symptoms on kale (Fig. 1) with different symptom severities. CO4 and CO2 isolates were the most virulent isolates (P = 0.05) (Table 1).

To characterize the genetic identity of *Rhizoctonia* species infecting kale with other binucleate *Rhizoctonia* spp., four kale isolates (CO1, CO2, CO3, CO4) were compared with 12 binucleate *Rhizoctonia* spp. testers available in our laboratory (AG A, AG Ba, AG Bb, AG Bo, AG C, AG D, AG F, AG G, AG O, AG P and AG Q), and 39 testers (13 binucleate *Rhizoctonia* spp. and 25 *R. solani*) publicly available sequences.

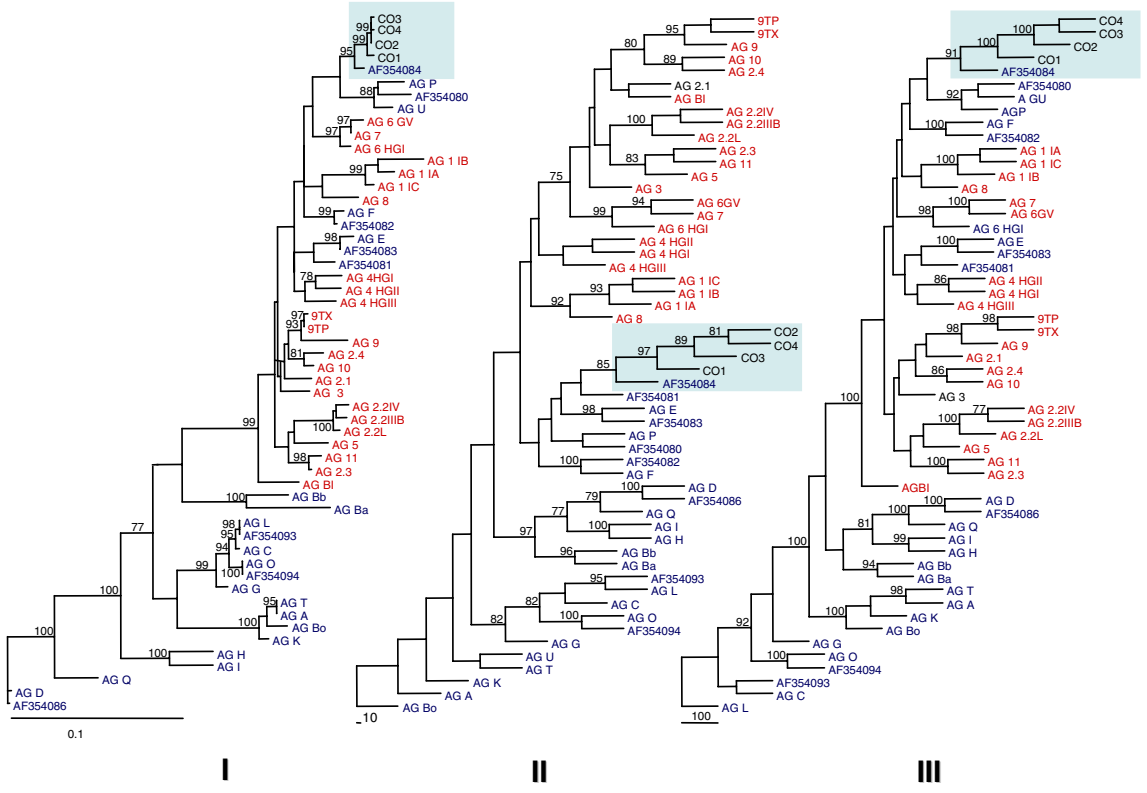

Fig. 2 Phylogenetic trees constructed by Maximum Likelihood (**I**), Maximum Parsimony (**II**), and Neighbour-Joining (**III**) methods illustrating the phylogenetic relationship of four isolates from kale (CO1, CO2, CO3, CO4) and 50 testers [26 binucleate *Rhizoctonia* spp. (blue colour), and 24 *R. solani* (red colour)]. The sequences of AF354086 (CAG1), AF354080 (CAG3), AF354081 (CAG4), AF354082 (CAG5), AF354083 (CAG6), AF354084 (CAG7), AF354093 (AGL), AF354094 (AGO), AB196663 (AGT) and AB196664 (AGU) are from GenBank. The numbers at branches indicate % of 100 bootstrap replications. Only branches with >75% are shown. The ITS1-5.8S-ITS2 sequences of *Rhizoctonia* binucleate spp. of kale were deposited at GenBank: CO1 (DQ279021), CO2 (DQ301760), CO3 (DQ279022) and CO4 (DQ301761)

Table 2 Similarity (%) between *Rhizoctonia* testers (*Rhizoctonia* binucleate species and *R. solani*) and isolates of kale (CO1, CO2, CO3, CO4)

Tester number	Isolate tester	CO1	CO2	CO3	CO4
AF354086 (CAG1)	*Rhizoctonia* binucleate	87.2	87.5	87.4	87.4
AG D	*Rhizoctonia* binucleate	87.2	87.5	87.4	87.4
AG Q	*Rhizoctonia* binucleate	88.3	87.9	87.7	87.7
AG Bb	*Rhizoctonia* binucleate	88.0	88.4	88.2	88.2
AG I	*Rhizoctonia* binucleate	88.2	88.4	88.2	88.2
AG Ba	*Rhizoctonia* binucleate	89.1	89.0	88.8	88.8
AG H	*Rhizoctonia* binucleate	89.4	89.6	89.4	89.4
AG T	*Rhizoctonia* binucleate	91.2	90.8	90.6	90.6
AG K	*Rhizoctonia* binucleate	91.0	90.8	90.6	90.6
AG A	*Rhizoctonia* binucleate	91.3	90.9	90.7	90.7
AG Bo	*Rhizoctonia* binucleate	91.3	91.4	91.3	91.3
AG G	*Rhizoctonia* binucleate	91.4	91.7	91.6	91.6
AG C	*Rhizoctonia* binucleate	92.1	92.4	92.2	92.2
AF354094 (AGO)	*Rhizoctonia* binucleate	92.2	92.4	92.2	92.2
AG O	*Rhizoctonia* binucleate	92.2	92.4	92.2	92.2
AF354093 (AGL)	*Rhizoctonia* binucleate	92.3	92.7	92.5	92.5
AG L	*Rhizoctonia* binucleate	92.3	92.7	92.5	92.5
AG 2.2IIIB	*Rhizoctonia solani*	92.7	92.7	92.5	92.5
AG 2.2IV	*Rhizoctonia solani*	92.7	92.7	92.5	92.5
AG 2-2LP	*Rhizoctonia solani*	93.2	93.2	93.0	93.0
AG 9	*Rhizoctonia solani*	93.0	93.2	93.0	93.0
AG 1 IB	*Rhizoctonia solani*	93.4	93.4	93.2	93.2
AG 2.4	*Rhizoctonia solani*	94.0	93.8	93.6	93.6
AG 1 IA	*Rhizoctonia solani*	93.8	94.0	93.8	93.8
AG BI	*Rhizoctonia solani*	94.4	94.0	93.8	93.8
AG 3	*Rhizoctonia solani*	94.7	94.5	94.3	94.3
AG 1 IC	*Rhizoctonia solani*	94.5	94.7	94.5	94.5
AG 11	*Rhizoctonia solani*	94.8	94.6	94.5	94.5
AG 5	*Rhizoctonia solani*	94.3	94.6	94.5	94.5
AG 4 HGI	*Rhizoctonia solani*	94.6	94.7	94.6	94.6
AG 4 HGII	*Rhizoctonia solani*	94.7	94.9	94.7	94.7
AG F	*Rhizoctonia solani*	94.9	94.9	94.7	94.7
AG 10	*Rhizoctonia solani*	94.7	94.9	94.7	94.7
9TX	*Rhizoctonia solani*	94.7	94.9	94.7	94.7
AG 2.3	*Rhizoctonia solani*	95.2	95.0	94.8	94.8
AG 4 HGIII	*Rhizoctonia solani*	95.1	95.1	94.9	94.9
9TP	*Rhizoctonia solani*	94.9	95.0	94.9	94.9
AF354082 (CAG5)	*Rhizoctonia* binucleate	95.3	95.3	95.1	95.1
AG 2.1	*Rhizoctonia solani*	95.1	95.2	95.1	95.1
AG 8	*Rhizoctonia solani*	95.3	95.4	95.2	95.2
AG 6GV	*Rhizoctonia solani*	95.2	95.4	95.2	95.2
AF354080 (CAG3)	*Rhizoctonia* binucleate	95.6	95.6	95.4	95.4
AG E	*Rhizoctonia* binucleate	95.6	95.6	95.5	95.5
AF354083 (CAG6)	*Rhizoctonia* binucleate	95.6	95.8	95.6	95.6

Table 2 continued

Tester number	Isolate tester	CO1	CO2	CO3	CO4
AG U	*Rhizoctonia* binucleate	95.9	95.9	95.7	95.7
AF354081 (CAG4)	*Rhizoctonia* binucleate	95.8	96.0	95.8	95.8
AG 7	*Rhizoctonia solani*	96.0	96.1	96.0	96.0
AG 6 HGI	*Rhizoctonia solani*	96.0	96.1	96.0	96.0
AG P	*Rhizoctonia* binucleate	96.4	96.4	96.2	96.2
AF354084 (CAG7)	*Rhizoctonia* binucleate	98.5	98.7	98.5	98.5
CO1		100	99.5	99.3	99.3
CO3		99.3	99.8	100	99.6
CO2		99.5	100	99.8	99.8
CO4		99.3	99.8	99.6	100

Total genomic DNA was extracted from the isolates using the methodology of Kuramae-Izioka (1997). The ITS4/ITS5 primer set (White et al. 1990) was used for PCR amplification of nuclear ITS1 and ITS2 regions and the 5.8S rRNA gene.

Each PCR product was purified using MicroSpin S-400 HR columns (Amersham Pharmacia), according to the instructions of the manufacturer, and sequenced using 10 ng of PCR product and 1 μM each of the ITS2, ITS3, ITS4 or ITS5 primers following the protocol supplied with the Amersham Premix Terminator (Amersham Pharmacia). Sequencing was performed using a PE Applied Biosystems Model 377 DNA Sequencer. The four sequenced fragments generated from each isolate were assembled using Phred/Phrap (Ewing et al. 1998) and Consed (Gordon et al. 1998). All consensus bases were of high quality with a Phred value >20. The consensus sequence for each isolate was trimmed in order to have the ITS1-5.8S-ITS2 sequence analyzed. First, the sequences were aligned using the computer software package CLUSTAL X (Thompson et al. 1997). The alignment parameters were gap opening = 10, gap extention = 0.20, delay divergent sequences = 30%, DNA transition weight = 0.50. Second, poorly aligned positions and divergent regions in the alignment were removed by using Gblocks 0.91b (Castresana 2000). The threshold parameters used were: minimum number of sequences for a conserved position = 50% of the number of sequences + 1, minimum number of sequences for a flank position = 85% of the number of sequences, maximum number of contiguous non-conserved positions = 8, minimum length of a block = 10. Thirty, distances (% divergence) between all pairs of sequences from the multiple alignment were calculated. The trees showing the phylogenetic relatedness between isolates and AG testers (*Rhizoctonia* binucleate and *R. solani*) were constructed using three different phylogenetic methods: maximum likelihood (PHYML) (Guindon and Gascuel 2003), maximum parsimony (MP), and neighbour-joining (NJ). For PHYML, the nucleotide model was HKY85 (Hasegawa et al. 1985), the transition/transversion ratio was 4, and the Gamma distribution parameter was 1.0. MP and NJ analyses were done using PROTPARS (heuristic search with characters equally weighted) and PROTDIST (Kimura formula) from Phylip (Felsenstein 1996), respectively. Non-parametric bootstrap support for MP and NJ was calculated from 100 resampling rounds.

All phylogenetic methods applied, viz. PHYML, MP, and NJ resulted in trees supporting the same phylogenetic position of the four kale isolates (Fig. 2). The kale isolates showed genetic identities ranging from 99.3 to 99.8% (Table 2) between them, even though they were collected from plants in the same field. The kale isolates were phylogenetically closely related to CAG 7 (AF354084) (Fig. 2), with identities of 98.5 and 98.7% (Table 2). The number of nucleotide differences among kale isolates and CAG 7 was higher at the ITS1 region (10 nucleotides) than at the ITS2 region (5 nucleotides). It is suggested that the binucleate *Rhizoctonia* spp. causing hypocotyl and root rot on kale Brazil comprises a new AG not yet described.

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