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Characteristics and diversity of *Rhizoctonia* spp. population in soil of selected forest bare-root nurseries in Poland

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Abstract

Fourty three *Rhizoctonia* isolates obtained from four forest nurseries situated in the Wielkopolska region (central-western Poland) has been proved as multinucleate (anamorph – *R. solani*). They represented four anastomosis groups (AG): AG1-IC, AG-5, AG4-HG2 and AG2-1. Three AGs were found in Jarocin nursery (AG-5, AG4-HG2 and AG2-1), two in Łopuchówko (AG-5 and AG4-HG2) and one in Konstantynowo (AG1-IC) and Pniewy (AG-5). All isolates were highly pathogenic to Scots pine (*Pinus sylvestris*) seedlings and pose a large damping-off threat to the seedlings in the nurseries with single AG and in those where more AGs exists.

Keywords: *Rhizoctonia* spp.; anastomosis group; pathogenicity; forest nursery; *Pinus sylvestris*; damping-off

Introduction

Coniferous species cover 69.9% of the total forest area in Poland, with Scots pine growing on 59.5% of the forest area of all forms of ownership [1]. The large percentage of pine stands in Polish forests requires constant renewal and therefore continuous production of large quantities of high-quality planting stock for reforestation is necessary.

Scots pine seedlings, both in nurseries and in natural stands, are vulnerable to attack by various pathogens [2–4].The most prevalent and severe disease of conifer seedlings is damping-off, the more frequently occurring, the longer a nursery is being operated, which is associated with accumulation of inoculum in soil [3,4].

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The most severe pathogens causing damping-off in Polish forest nurseries include species from genera: *Fusarium*, *Rhizoctonia*, *Pythium*, *Alternaria* and *Cylindrocarpon* [3,5–8]. Fungi belonging to the *Rhizoctonia* genus represent a wide range of pathogenic, non-pathogenic and mutualistic species [9]. Most studies on this group of fungi, known to attack more than 200 different species of plants, relate to agricultural plants, while only few researchers have studied this group of organisms in forest nurseries [4,6,10–22].

Fungi classified as *Rhizoctonia* spp. are a collection of fungi, in which the differences relate to the stage of anamorph, teleomorph, size and shape of sclerotia, and color of the mycelium [23–30].

The first reports on anastomosis reactions between isolates of *R. solani* date back to 1920 and 1930 [31,32]. However, only since the emergence of the anastomosis groups (AGs) concept within *Rhizoctonia* spp. [33], it has become an important tool in understanding the genetic diversity of this complex of fungi, considering the fact that isolates subscribed to different anastomosis groups can differ in pathogenicity to their host plants [34].

Although *Rhizoctonia* spp. are an important problem in a number of forest nurseries in Poland but until recently, there has been little research in this area. The fact that hyphal anastomosis groups in *Rhizoctonia* spp. are not always host specific is well known and most isolates from different anastomosis groups are capable of causing infection on a variety of plant species [24]. Because virulence and host range of AGs is differentiated, therefore knowledge about affinity of isolates of *R. solani* to particular anastomosis group is very important, in view of protection of Scots pine seedlings not only in Wielkopolska region but in the entire country.

The aim of this study was to investigate the inter- and intra-species diversity within *Rhizoctonia* spp. isolated from soil of four selected forest nurseries in the Poznań region (central-western Poland) in connection with their pathogenicity diversity and the resulting threat to Scots pine seedlings in Polish nurseries.

Material and methods

Collecting isolates

Soil samples were harvested from the top 15 cm of the soil profile from nurseries of Forest Districts: Jarocin, Łopuchówko, Konstantynowo and Pniewy. The soil was collected in early spring, before any chemicals were used. Samples were placed in sterile cotton bags and stored at 5°C for no longer than two days.

Rhizoctonia isolates were obtained from soil samples using two trapping methods. The first method based on the use of pine seeds which were sown (25 seeds per pot) to a volume of 700 ml of unified soil from a nursery. Then, after emergence of the first seedlings observation was carried out in order to capture the very first symptoms of damping-off. The symptomatic seedlings were disinfected with 0.5% sodium hypochlorite for 5 minutes, 70% ethanol (1 minute) and washed three times in distilled sterilized water, for five minutes jointly. Next, the symptomatic parts of seedlings (up to 2 cm) were transferred onto Martin-Johnson agar medium, supplemented with antibiotics (streptomycin 0.05 g/l and aureomycin 0.05 g/l) to prevent the growth of bacteria. All isolates growing out of them were transferred onto potato dextrose agar (PDA medium).

The second method used in the study was a modified method described by Paulitz and Schroeder [35] where wooden toothpicks were used. Toothpics (10 per 700 ml of soil in pot) were inserted into the soil to a depth of 5 cm, evenly spaced in the pot. After 48 hours, toothpicks were removed and placed on Petri plates with PDA, supplemented with antibiotics (as described above). The plates with two toothpics per plate were incubated for 24 hours at room temperature. All mycelia growing from tothpicks were transferred onto PDA plates in order to identify *Rhizoctonia* isolates.

***Rhizoctonia* identification**

MICROSCOPIC EXAMINATION BY CELL NUCLEI STAINING. Determining the number of nuclei in the cells of the *Rhizoctonia* spp. is an important part of the process of assigning isolates to this genus [28].

The number of nuclei per cell was determined with the method described by Bandoni [36]. The average number of nuclei was calculated for 50 cells of each isolate.

MOLECULAR IDENTIFICATION. **DNA isolation.** In order to isolate DNA, isolates were grown on liquid broth for 6–8 days. After that time the mycelium was placed on sterile filter paper and the excessive medium was removed. The dried mycelium was put into Eppendorf tubes (1.2 ml), and placed for 24 hours at −20°C. The next step was freeze-drying of the frozen mycelia in a lyophilizer (Christ Alpha 1-2 LD) at −55°C and a pressure of 10^{-2} bar. After at least 18 hours, the mycelium was crushed with metal rods, then the cap was secured with parafilm, and the tubes were stored at −20°C until the DNA extraction. DNA extraction was carried out using a DNeasy Plant Mini Kit (Qiagen) according to manufacturer's recommendations. **PCR-RFLP analysis of rDNA-ITS region.** Isolates obtained in the study have been first separated based on morphological differences and the number of nuclei per cell. In order to assign isolates to the anastomosis subgroups the restriction enzymes digestion of RFLP of rDNA-ITS regions was performed. Primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) were used for amplification of the nuclear rDNA-ITS region [37]. The PCR amplification reactions were conducted by adding 2 μl genomic DNA to 23 μl of PCR-mix, containing 2.5 μl 10× PCR buffer (Qiagen), 5 μl Q-solution (Qiagen), 0.5 μl dNTPs (10 mm, Fermentas GmbH), 1.75 μl of each primer (10 μm), 0.15 μl Taq polymerase (Fermentas GmbH) and 11.35 μl sterile highly purified water. The denaturation step at 94°C for 10 min was followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. Cycling ended with a final extension step at 72°C for 10 min (Biometra, T-Gradient Thermoblock). After the DNA extraction and restriction enzymes digestion of RFLP of rDNA-ITS regions the results of electrophoresis were checked under the UV light, and the resulting patterns were compared with those presented in the work of Guillemaut et al. [38]. Four restriction enzymes (MseI, AvaII, HincII, and MunI) were used in the study. **Sequence analysis of ITS-rDNA region.** Sequencing of ITS-rDNA region and previous cleaning of the DNA has been carried out in the Laboratory of Medical Genetics, CB DNA (Mickiewicza 31, 60-835 Poznań). The sequencing results were then used for further work. The sequences of rDNA-ITS region were compared with sequences available in the GenBank (http://www.ncbi.nlm.nih.gov/GenBank) to check and compare if the results of those carried with Guillemault et al. [38] method were the same.

CHARACTERISTICS OF *RHIZOCTONIA* ISOLATES. **Growth rate measurement.** Fungal colonies were grown at 21°C, according to the work of Tewoldemedhin et al. [39].

The mycelial disc (0.5 cm diameter) of each isolate (4 reps) was placed in the center of Petri dishes with PDA. The Petri dishes were incubated in the dark. The growth rate of individual isolates on PDA was measured every 24 hours, in two directions on each plate.
Pathogenicity of *Rhizoctonia* isolates. Plastic pots (500 ml volume) were filled up to 2/3 with sterilized soil (120°C for 2 hours, for three days in a row). On the soil surface PDA medium overgrown with seven-day-old mycelium removed from a Petri dish was placed and covered with a further layer of soil (about 1 cm) into which the Scots pine seeds were sown (25 seeds per pot). Seeds were previously surface sterilized for 10 minutes in 30% H_2O_2, then rinsed with distilled water three times. Thus prepared pots, and the control pots (with sterile PDA medium) were watered with tap water as needed. After the first seedlings were observed emerging from the soil, the pots were checked daily and infested plants were counted. For each isolate four reps were carried out.

Results

Collected isolates

Of all the soil samples collected from the surveyed nurseries 43 isolates (Tab. 1) classified as *Rhizoctonia* were obtained, 24 isolates from forest nursery belonging to Jarocin Forest District, 8 from Łopuchówko, 7 from Pniewy and 4 from Konstantynowo.

All the isolates were multinucleate, that is representing *R. solani* anamorph. No binucleate or uninucleate *Rhizoctonia* were found in the surveyed nurseries. The lowest average number of nuclei was observed in AG1-IC isolates obtained from Konstantynowo (Tab. 2). The highest number of nuclei per cell was observed in AG-5 isolates obtained from Jarocin, with the average number of 9.5 nuclei per cell (AG-5 isolates from Łopuchówko and Pniewy average 8.853 and 8.637 nuclei per cell, accordingly). Rhizoctonia AG2-1 was characterized by the average number of 6.02 nuclei per cell while AG4-HG2 by 5.5 and 7.268 nuclei per cell (Jarocin and Łopuchówko, accordingly).

Characteristics of *Rhizoctonia* isolates

MYCELIUM GROWTH RATE. On average, isolates belonging to AG-5 were characterized by the fastest growth within all isolates (13.9 mm/24 h), and the slowest growing were those assigned to AG4-HG2 (8.91 mm/day). The greatest differences in growth were observed within AG-5, and the least within AG4-HG2 (Fig. 1).

PATHOGENICITY OF RHIZOCTONIA ISOLATES. Differentiation within anastomosis groups in terms of pathogenicity was statistically significant for all nurseries (at a confidence level of 0.001; Fig. 2). All isolates, regardless of the place of origin of the individual anastomosis groups, were characterized by high pathogenicity to pine seedlings.

The most pathogenic isolates proved those belonging to AG-5 isolated from Łopuchówko (100% mortality). The least pathogenic ones were found to be AG4-HGII isolates, wherein the average pathogenicity of all the isolates tested was 92.81% (Fig. 2).

ANASTOMOSIS GROUPS. *Rhizoctonia* isolates obtained in the study have been first separated based on morphological differences and the number of nuclei per cell. After the DNA extraction and restriction enzymes digestion of RFLP of rDNA-ITS regions the results of electrophoresis were checked under the UV light, and the resulting patterns were compared with those presented in the work of Guillemaut et al. [38] (Fig. 2).

Tab. 1 Anastomosis groups and species of *Rhizoctonia* isolates studied.

No.	Isolate	Anamorph	Teleomorph	Anastomosis group/subset	Accession No.	Percentage sequence simillarity
Konstantynowo						
1	140712	*R. solani*	*T. cucumeris*	AG1-IC	FJ746941.1	99
2	140711	*R. solani*	*T. cucumeris*	AG1-IC	FJ746941.1	99
3	140707	*R. solani*	*T. cucumeris*	AG1-IC	FJ746941.1	99
4	140729	*R. solani*	*T. cucumeris*	AG1-IC	FJ746941.1	99
Jarocin						
5	130750	*R. solani*	*T. cucumeris*	AG-5	GU055587.1	99
6	130711	*R. solani*	*T. cucumeris*	AG-5	GU055587.1	99
7	130724	*R. solani*	*T. practicola*	AG4-HG2	AB000032.1	99
8	130725	*R. solani*	*T. practicola*	AG4-HG2	AB000032.1	99
9	130728	*R. solani*	*T. practicola*	AG4-HG2	AB000032.1	99
10	130714	*R. solani*	*T. practicola*	AG4-HG2	AB000032.1	99
11	130704	*R. solani*	*T. practicola*	AG4-HG2	AB000034.1	99
12	130703	*R. solani*	*T. practicola*	AG4-HG2	AB000034.1	99
13	130733	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
14	130710	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
15	130734	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
16	130712	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
17	130707	*R. solani*	*T. cucumeris*	AG2-1	FJ492102.3	99
18	130745	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
19	130752	*R. solani*	*T. cucumeris*	AG2-1	FJ492102.3	99
20	130718	*R. solani*	*T. cucumeris*	AG2-1	FJ492102.3	99
21	130744	*R. solani*	*T. cucumeris*	AG2-1	AB054853.1	99
22	130713	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
23	130723	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
24	130729	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
25	130772	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
26	130764	*R. solani*	*T. cucumeris*	AG2-1	AY154317.1	99
27	130765	*R. solani*	*T. cucumeris*	AG2-1	FJ492102.3	99
28	130794	*R. solani*	*T. cucumeris*	AG2-1	FJ492102.3	99
Łopuchówko						
29	150711	*R. solani*	*T. practicola*	AG4-HG2	AB000020.1	99
30	150708	*R. solani*	*T. practicola*	AG4-HG2	AB000020.1	99
31	150703	*R. solani*	*T. practicola*	AG4-HG2	HQ629864.1	99
32	150710	*R. solani*	*T. practicola*	AG4-HG2	AB000020.1	99
33	150712	*R. solani*	*T. practicola*	AG4-HG2	HQ629864.1	99
34	150702	*R. solani*	*T. cucumeris*	AG-5	EU244843.1	99
35	150707	*R. solani*	*T. cucumeris*	AG-5	EU244843.1	99
36	150709	*R. solani*	*T. cucumeris*	AG-5	EU244843.1	99

Tab. 1 *(continued)*

No.	Isolate	Anamorph	Teleomorph	Anastomosis group/subset	Accession No.	Percentage sequence simillarity
Pniewy						
37	160710	*R. solani*	*T. cucumeris*	AG-5	HQ898767.1	99
38	160702	*R. solani*	*T. cucumeris*	AG-5	HQ629863.1	99
39	160704	*R. solani*	*T. cucumeris*	AG-5	HQ629863.1	99
40	160712	*R. solani*	*T. cucumeris*	AG-5	HQ898767.1	99
41	160703	*R. solani*	*T. cucumeris*	AG-5	HQ898767.1	99
42	160729	*R. solani*	*T. cucumeris*	AG-5	HQ898767.1	99
43	160705	*R. solani*	*T. cucumeris*	AG-5	HQ898767.1	99

Tab. 2 The average number of nuclei per cell in different anastomosis groups (mean value and standard deviation – s.d.; univariate analysis followed by NIR test was carried out only for two nurseries).

Anastomosis group	Jarocin		Konstantynowo		Łopuchówko		Pniewy	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
AG-5	9.50a	0.707			8.853a	1.263	8.637	0.989
AG1-IC			4.700	0.294				
AG2-1	6.02b	0.830						
AG4-HG2	5.50b	0.775			7.268a	1.345		
$NIR_{0.05}$	1.69				2.36			
$P > F$	<0.001				0.151			

On this basis, the isolates were assigned to particular anastomosis groups. It has also been verified by comparing the ITS-rDNA sequences of all isolates with sequences deposited in GenBank (Tab. 1).

Isolates assigned to AG1-IC have only been found in Konstantynowo. They were characterized by the largest similarity to the isolates deposited in the GenBank database under FJ746941.1 accession number. The isolate deposited in GenBank has been isolated from zoysiagrass (*Zoysia* sp.) where it has been a causal agent of large patch disease.

Jarocin's isolates assigned to AG-5 showed a high homology (99%) with isolate GU055587.1, obtained from agricultural soil from Lower Austria. AG-5 isolate obtained from Łopuchówko was similar in 99% to those obtained from Switzerland (EU244843.1), whereas those obtained from Pniewy showed high homology with isolates HQ898767.1, associated with potato tubers in France, and isolate HQ629863.1 affecting *Pisum sativum* in North Dakota.

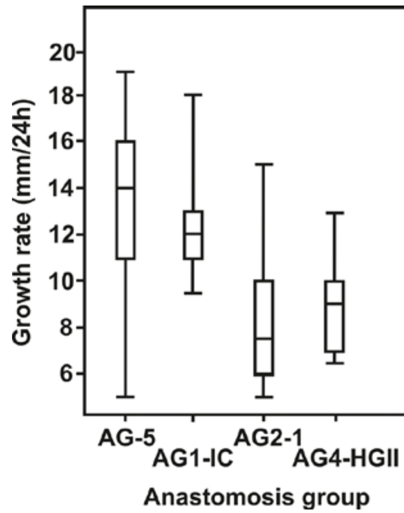

Fig. 1 Growth rate of different anastomosis groups of *Rhizoctonia* spp. used in the study (Box-and-Whisker Plot: upper whisk – highest value; upper quartile; median; lower quartile; lower whisk – lowest value).

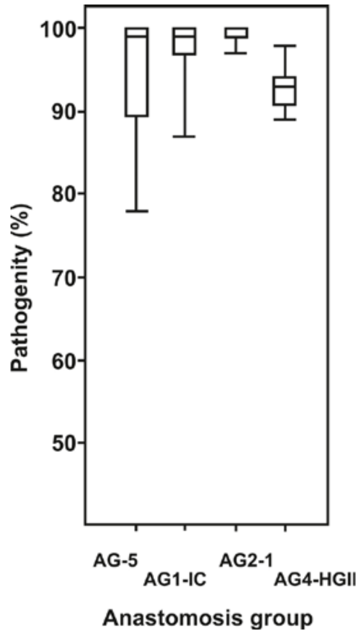

Fig. 2 Pathogenicity of isolates of *Rhizoctonia* spp. belonging to different anastomosis groups (Box-and-Whisker Plot: upper whisk – highest value; upper quartile; median; lower quartile; lower whisk – lowest value).

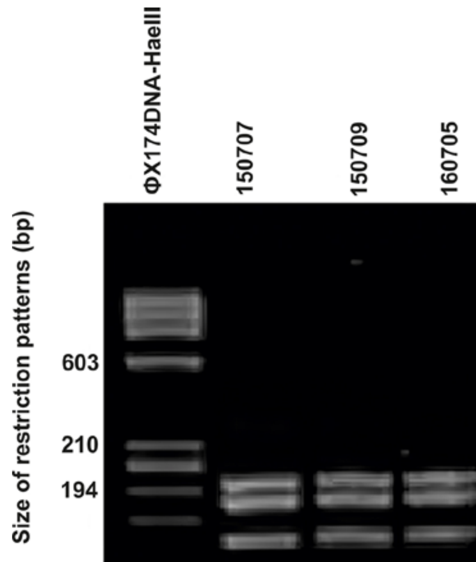

Fig. 3 Example of RFLP analysis of the ITS rDNA region for isolates of 150707, 150709 and 160705 (AG-5) of *Rhizoctonia solani* amplified with primers ITS4 and ITS5 and digested with MunI.

Isolates described in this work as AG4-HG2 were found in two nurseries – Jarocin and Łopuchówko. From among AG4-HG2 isolates from Jarocin four were highly similar to AB000032.1, whereas two were more similar to AB000034.1. Both sequences deposited in GenBank were obtained from Inner Mongolia. Isolates from Łopuchówko were most similar to HQ629864.1 isolated from *Pisum sativum* in North Dakota (isolates 150703 and 150712) and AB000020.1 isolated from potato in Inner Mongolia (isolates 150708, 150710, 150711). The biggest number of isolates (16) obtained in this study belonged to AG2-1. All of them were isolated from Jarocin. They were highly homological with AY154317.1, FJ492102.3 and AB054853.1 deposited in GenBank. Unfortunately, in the GenBank database there is no information from what plant isolate AY154317.1 has been obtained. The isolate FJ492102,3 was isolated from sugar beet in the USA. Isolate deposited in the GenBank under the number AB054853.1 has been obtained in Italy from *Nicotiana tabacum*.

Discussion

In this study, 43 *Rhizoctonia* isolates were obtained from four forest nurseries and their anastomosis groups (AGs) and anastomosis subgroups were determined. They all proved to be *R. solani*, a species widely distributed in Polish forest nurseries. Our understanding concerning the occurrence of *Rhizoctonia* anastomosis groups in Polish forest nurseries is very scarce. Knowledge of *Rhizoctonia* spp. AGs occurring in the nursery could facilitate the selection of plant protection methods and products to protect the seedlings against

these pathogens. The results of some studies [40–42] point to different sensitivity to fungicides of isolates belonging to different AGs. On the other hand, the works of Herr [43] and Sharon et al. [44] suggest the possibility of using non-pathogenic strains as biological control agents against highly pathogenic strains of *Rhizoctonia*.

Pathogenicity tests of different AGs to Scots pine and Norway spruce (*Picea abies*) carried out on uninucleate isolates confirm that they are the causing agents of damping-off in many Finnish nurseries [45–47]. At the same time from Norwegian and Finnish forest nurseries uninucleate and binucleate strains of *Rhizoctonia* have been isolated from healthy seedlings of Scots pine and Norway spruce [48–51]. So far, no uninucleate isolates were obtained from Polish forest nurseries.

In one of the few works on *Rhizoctonia* spp. in forest nurseries Camporota and Perrin [52] have demonstrated the predominant role of *R. solani* as the primary pathogen causing damping-off of pine seedlings. This is also confirmed by our study where all the obtained isolates proved to be multinucleate. So far, 14 anastomosis groups (AG-1 to AG-13 and AG-B1) have been described within multinucleate *Rhizoctonia* [53]. Eight groups (AG-1 to AG-6, AG-8 and AG-9) were further subdivided into subgroups. From among those Stępniewska-Jarosz et al. [6] found five different anastomosis groups in Polish forest nurseries. The most frequent was AG-5 (37% of isolates), followed by AG 2-1 (30%) and 27% of the isolates were identified as AG-4. Groups AG 1-IB and AG 2-2 were only represented by single isolates. In our study one of the nurseries (forest nursery Jarocin) examined earlier by Stępniewska-Jarosz et al. [6], was surveyed for *Rhizoctonia* spp. for the second time and no binucleate isolates were found. Two AGs found in the previous study were also isolated: AG-5 (2 isolates) and AG 2-1 (16 isolates), while the AG4-HG2 (6 isolates) has not been previously observed. The difference may result from possible bridging of some isolates belonging to certain AGs with isolates belonging to the other [24] in the meantime and/or from the fact that different techniques were used to assign isolates to certain AGs. While Stępniewska-Jarosz et al. [6] used classic techniques, with culture of isolates in vitro and subsequent observation of their growth under microscope, our study was fully based on molecular methods.

Though the anastomosis groups differed significantly from each other in terms of pathogenicity, all the isolates studied proved to be highly pathogenic to Scots pine seedlings. This means that the AGs in question may contribute to considerable damping-off threat to the seedlings in all the nurseries.

As most of the research on *Rhizoctonia* in Poland has been conducted on agricultural plants, it is highly desirable to further screen forest nurseries for the pathogen as well, both for better recognition of the situation and for the hope of finding non-pathogenic or mutualistic species, which could possibly contribute to decreasing the infection threat.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: collected material, laboratory works, photographs, statistics, bibliography, draft of the manuscript: MB; helped writing the manuscript, critical revising: MM.

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