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Waitea circinata var. *zeae* Causing Root Rot of Cabbage and Oilseed Rape

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Abstract

Cabbage, a widely used and popular vegetable, and oilseed rape, the second most valuable oilseed crop in the world, are two important species from the Brassicaceae family. Two geographically separated outbreaks of cabbage and oilseed rape root rot with estimated incidence of 15 and 20%, respectively, were recorded during 2017 in the Vojvodina region, Serbia. Twelve hyphal-tip isolates were obtained from symptomatic cabbage and oilseed rape plants and identified as *Waitea circinata* var. *zeae* based on morphological and molecular features. This indicates that *W. circinata* var. *zeae* has expanded its host range to the Brassicaceae family. Sequence analyses of internal transcribed spacer (ITS) and large subunit of the ribosomal DNA, *RPB2,* and β-*tubulin* genes revealed the highest similarity with multiple *W. circinata* var. *zeae*. Neighbor-joining analyses of ITS sequences resulted in a phylogenetic tree with one well-defined branch of *W. circinata* var. *zeae,* with two separate groups. All Serbian isolates and the majority of isolates originating from natural infection of dicotyledonous plants grouped together in group I. Following artificial inoculation, *W. circinata* var. *zeae* isolates caused mild to medium root necrosis of seedlings of 2 monocotyledonous and 12 dicotyledonous plant species, implying a wider host range than was known for *W. circinata* var. *zeae.* Additionally, this is the first occurrence of *W. circinata* var. *zeae* on dicotyledonous host plants in Europe. Because cabbage and oilseed rape are important crops grown worldwide, the occurrence of this new soilborne pathogen with a broad host range imposes the necessity for changes in routine disease control practices, particularly crop rotation.

Keywords: field crops, fungi, oilseeds and legumes, pathogen detection, pathogen diversity

Destructive, soilborne plant pathogens from the genus *Rhizoctonia* are the causal agents of damping off, root and stem rot, and blight of many important crops. As a complex genus without a fully resolved concept of species, the genus *Rhizoctonia* comprises multinucleate, binucleate, and uninucleate species according to the number of nuclei in their hyphae (Ogoshi 1996). Based on hyphal anastomosis, both multinucleate (associated with teleomorphs of *Thanatephorus* and *Waitea*) and binucleate (teleomorphs of *Ceratobasidium* and *Tulasnella*) isolates are further separated into numerous anastomosis groups (AGs) (Ogoshi 1987).

Multinucleate species associated with the teleomorph *Waitea circinata* Warcup & P. H. B. Talbot were reported for the first time from soil in Australia in the 1960s. Initially, three varieties were described: *W. circinata* var. c*ircinata*, *W. circinata* var. *zeae* (associated with *Rhizoctonia zeae* anamorphic isolates), and *W. circinata* var. *oryzae* (anamorphic isolates *R. oryzae*) (Leiner and Carling 1994). More recently, also based on the colony morphology, two additional varieties have been described; namely, *W. circinata* var. *agrotis* (Toda et al. 2007) and *W. circinata* var. *prodigus* (Kammerer et al. 2011). Usually, a reliable identification of *Waitea* spp. can be achieved by the combination of conventional AG pairing and sequencing of internal transcribed (ITS) region ITS1, 5.8S ribosomal DNA (rDNA), and ITS2 regions of the rDNA (Fang et al. 2013; Li et al. 2011; Manici and Bonora 2007; Martin 2000; Sharon et al. 2007, 2008), while biochemical and molecular analyses, including phylogenetic analyses of the ITS region (Aydın et al. 2013; de la Cerda et al. 2007; Kammerer et al. 2011; Sharon et al. 2006; Toda et al. 2007) or amplified fragment length polymorphism (El Fiky et al. 2011), are sufficient for differentiating between varieties.

Although less frequently isolated compared with other multinucleate and binucleate *Rhizoctonia* spp., *W. circinata* varieties have been isolated from symptomatic plants or from agricultural soil in different parts of the world; namely, Japan (Toda et al. 2005, 2007), Korea (Chang and Lee 2016), Australia (Lanoiselet et al. 2011), Canada (de la Cerda et al. 2007), the United States (Amaradasa et al. 2013; Chen et al. 2009; Kammerer et al. 2011;Leiner and Carling 1994; Paulitz et al. 2003), Brazil (Blanco et al. 2018; Chavarro-Mesa et al. 2020), South Africa (Tewoldemedhin et al. 2015), Turkey (Demirci 1998; Demirci and Eken 1999; Erper et al. 2005), and, more recently, Hungary (Vajna and Oros 2005) and Spain (Gómez de Barreda et al. 2019) in Europe. Among *W. circinata* varieties, var. *zeae* is the most common (Blanco et al. 2018) and probably has the broadest host range mainly, oriented to maize, wheat, barley, warm- and cool- season turfgrasses, and other host plants from the family Poaceae (Martin and Lucas 1984; Oros et al. 2013; Sumner and Bell 1982), and onion from the family Liliaceaae (Erper et al. 2006). Additionally, some of the *W. circinata* var. *zeae* isolates have recently been isolated as causal agents of root rot of dicotyledonous agricultural plants, including bean, soybean, pea, and rooibos (Fabaceae) (Erper et al. 2005, 2011; Ohkura et al. 2009; Sharma-Poudyal et al. 2015; Tewoldemedhin et al. 2015), carrot (Apiaceae) (Ohkura et al. 2009), and sugar beet (Chenopodiaceae) (Kuznia and Windels 1994; Zhao et al. 2019). Nonpathogenic isolates of *W. circinata* var. *zeae* have also been recovered from symptomatic tobacco plants (Mercado Cárdenas et al. 2015). In addition, isolates of unconfirmed pathogenicity have been recovered from various agricultural soils (Leiner and Carling 1994; Tomaso-Peterson and Trevathan 2007) and from soil where the previous crops were coffee (Blanco et al. 2018) and soybean (Ploetz et al. 1985).

Managing diseases caused by soilborne pathogens from the genus *Rhizoctonia* is difficult due to several obstacles such as the lack of fungicides with mode of action against *Rhizoctonia*, economically inefficient fungicide treatments, the pathogen long-term persistence in soil, and frequent overlapping of host range between species and AGs (Allen et al. 1985; Kiewnick et al. 2001). Novel approaches, including compost soil amendments (Tewoldemedhin et al. 2015) and

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biological control using nonpathogenic *R. zeae* isolates (Webb et al. 2015), are promising but have to be supported by targeted research of a particular geographic location. Considering the known differences among AGs in host range and fungicide sensitivity, reliable and fast detection and identification of *Rhizoctonia* AGs associated with symptomatic plants is of the greatest importance (Amaradasa et al. 2013; Buhre et al. 2009; Lakshman et al. 2016).

In this article, we present the first record of *Rhizoctonia*-like isolates causing root rot of cabbage (*Brassica oleracea* var. *capitata*) and oilseed rape (*B. napus* var. *oleifera*) in Serbia. This finding is important because oilseed rape is the second most important oilseed crop in the world, with the European Union being the fourth largest producer (Carre and Pouzet 2014). At the same time, cabbage is among the most popular vegetables in the world, with China being the biggest producer while Europe and Russia are its biggest consumers (FAOSTAT; http://www.fao.org/faostat/en/#data/QC). Oilseed rape production in Serbia is among the fastest growing, and has almost tripled in size during only 2016 to 2018 (https://www.stat.gov.rs/). On the other hand, cabbage is among the most important and readily consumed vegetables in Serbia, with stable annual production on over 10,000 ha https://www.stat.gov.rs/). The occurrence of root rot of cabbage and oilseed rape in Serbia during 2017 raised a concern among the growers, particularly because crop rotation with crops described as susceptible in literature is a regularly applied disease control measure. The main objectives of this study were to (i) identify and characterize selected isolates based on morphological features and AG pairing; (ii) determine their taxonomic position based on sequencing of four genetic markers, including ITS (ITS1, 5.8S rDNA, and ITS2) and large subunit (LSU) of the rDNA, *RNA polymerase II* (*RPB2*) gene, and the protein coding gene β-*tubulin*; (iii) explore phylogenetic relationships among *W. circinata* isolates, with emphasis on *W. circinata* var. *zeae* isolates originating from dicotyledonous plants; and (iv) test their pathogenicity and aggressiveness by artificial inoculation of a wide range of possible host plants.

Materials and Methods

Sample collection and isolation. During 2017, samples (n = 43) of oilseed rape and cabbage with the symptoms of root rot were collected from two distinct localities in the Vojvodina District, Serbia. At the locality of Futog, 19 samples of symptomatic cabbage plants Srpski melez were collected, while at the locality Rimski Šančevi, 24 samples of symptomatic oilseed rape NS-Zorica were collected. Plants were in the phenophase of two to six true leaves. Disease incidence was calculated by randomly counting and rating 100 plants in four replications. At both localities, plants exhibiting wilting symptoms were randomly sampled by zigzag walking throughout the fields. From each sample, small tissue fragments from the border between necrotic and healthy tissue were surface sterilized with 2% sodium hypochlorite, placed on the potato dextrose agar (PDA; 200 g of potato, 20 g of dextrose, 17 g of agar, and 1 liter of distilled H_2O) and incubated at 24°C for 3 to 5 days. From *Rhizoctonia*-like colonies, hyphal-tip isolates were obtained and their pathogenicity (the ability to cause disease on a respective host plant) was tested by artificial inoculation of cabbage and oilseed rape seedlings. The isolates are maintained on sealed PDA slants at 4°C in the Fungal Collection of the Department of Phytopathology, Institute of Phytomedicine, University of Belgrade–Faculty of Agriculture, and are available for scientific exchange on request.

In total, 12 isolates (6 isolated from cabbage and 6 from oilseed rape, all originating from different locations of sampled fields) were used for morphological identification, AG pairing, and ITS sequencing, while 2 isolates (299-17 from cabbage and 300-17 from oilseed rape) were selected for sequencing of LSU, *RPB2*, and β-*tubulin* genomic regions, assessment of temperature requirements, and host range pathogenicity and aggressiveness (the ability to infect and cause different intensity of the disease) trials (Table 1).

Morphological identification. Morphological identification of *Rhizoctonia*-like isolates was based on colony appearance; hyphal branching pattern; the presence, distribution, and color and dimensions of sclerotia; and the number of nuclei in cells of young hyphae (Ogoshi 1987). Colony morphology was assessed 15 days postinoculation (dpi) on PDA at 24°C in darkness. The hyphal branching pattern was microscopically observed in cultures using a compound microscope (Olympus CX41; Olympus Europa SE & Co. KG).

The number of nuclei per cell was determined using the clean-slide technique (Kronland and Stanghellini 1988) combined with staining with aniline blue in lactophenol or safranin O (Herr 1979), followed by direct observation using dark-field microscopy. To confirm the nuclear status of each isolate, nuclei in 20 young hyphal cells per isolate on at least five different slides were counted.

Hyphal anastomosis reaction. Hyphal anastomosis reaction was assessed by pairing *Waitea* isolates (Table 1) with each other and with the available tester isolates of binucleate AG-D (isolate R13-1) and multinucleate AG-1-IC (R62), AG-2-1 (00269), AG-2-2 (01336), AG-3 (R14 1/97 T1), AG-4-HGII (2319), AG-5 (B8), AG-6 (06-01), AG-8 (R28), and AG-9 (CBS970.96) *Rhizoctonia* isolates, using the modified clean-slide technique (Kronland and Stanghellini 1988).

Temperature requirement assessment. In order to determine the temperature range for minimal and optimal growth, two selected *Waitea* isolates (299-17 and 300-17, originating from cabbage and oilseed rape, respectively) were grown on PDA and incubated in darkness at the following range of temperatures: 5, 10, 15, 20, 25, 30, 35, and 40°C. The average growth rate was calculated based on colony diameters in five replications and the experiment was repeated twice.

DNA amplification and sequencing. Total genomic DNA was extracted from 100 mg of dry mycelium sampled from 5- to 7-day-old cultures of 12 *Waitea* isolates (Table 1) grown on potato dextrose broth (200 g of potato, 20 g of dextrose, and 1 liter of distilled H_2O) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR amplification of four markers, including ITS (ITS1, 5.8S rDNA, and ITS2) and LSU of the rDNA, *RPB2,* and β-*tubulin* regions were performed in a total reaction volume of 25 μl, consisting of 12.5 μl of 2× PCR Master mix (Fermentas, Lithuania), 9 μl of RNase-free water, 2.5 μl of both forward and reverse primers (working solution with a final concentration of 100 pmol/μl) (Metabion International, Deutschland), and 1 μl of template DNA. Amplification conditions (Table 2) were as follows: initial denaturation at 94°C for 3 min; followed by 35 to 40 cycles of denaturation at 95°C for 30 s, variable duration and annealing temperatures, and elongation at 72°C for 1 min; and final elongation for 10 min at 72°C. The obtained amplicons were stained with ethidium bromide, analyzed by 1% agarose gel electrophoresis, and visualized using a UV transilluminator. The PCR products of all genomic regions were directly sequenced in both directions using an automated sequencer (ABI 3730XL Automatic Sequencer; Macrogen Inc., Korea), using the same primers as for amplification. Consensus sequences were computed using ClustalW (Thompson et al. 1994), integrated in MEGA6 software (Tamura et al. 2013), and deposited in GenBank (http://www.ncbi.nlm.nih.gov). All generated sequences were compared with each other by calculating nucleotide similarities, as well as with previously deposited *Rhizoctonia* spp. isolates available in GenBank, using the similarity search tool BLAST.

Phylogenetic analyses. ITS sequences generated from 12 selected Serbian *Waitea* isolates from cabbage and oilseed rape were analyzed with 31 previously listed type-derived sequences of *Waitea* spp. (Aydın et al. 2013) and one outgroup taxa multinuclear *Rhizoctonia solani* AG-1 IB (Aktaruzzaman et al. 2015). Of the 32 sequences retrieved from GenBank, 21 were of either *W. circinata* var. *zeae* associated with dicotyledonous plants or selected isolates originating from the main geographic areas of distribution. The remaining 11 sequences were representative sequences of other *W. circinata* varieties and outgroup *R. solani* AG-1 IB (Table 3). A phylogenetic tree was inferred using the neighbor-joining method (Saitou and Nei 1987) implemented in MEGA 6.0 software (Tamura et al. 2013). Distances in the ITS rDNA region were determined using Kimura's

two-parameter model (Kimura 1980), and all sites with gaps were omitted. The reliability of the obtained trees was evaluated using 1,000 bootstrap replicates.

Pathogenicity, experimental host range, and aggressiveness testing. Pathogenicity of all 12 isolates was tested on the five seedlings of oilseed rape or cabbage, depending on their origin. Seedlings were inoculated in the phenophase of first true leaves by placing five mycelial plugs (2r = 5 mm) from 7-day-old cultures of each isolate near roots during the planting. Five seedling were inoculated with each isolate, while seedlings inoculated with sterile PDA served as control. Plants were maintained under glasshouse conditions, and presence of symptoms was recorded 7 dpi. Reisolations were caried out from all symptomatic seedlings using the same methods as for initial isolations.

The experimental host range and aggressiveness of two isolates (299-17 and 300-17) (Table 1) was assessed by artificial inoculation of 2 monocotyledonous and 12 dicotyledonous hosts: wheat (*Triticum aestivum*), maize (*Zea mays*) (Poaceae), cabbage, oilseed rape (Brassicaceae), tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), tobacco (*Nicotiana tabacum*) (Solanaceae), cucumber (*Cucumis sativus*) (Cucurbitaceae), lettuce (*Lactuca sativa*), sunflower (*Helianthus annuus*) (Asteraceae), pea (*Pisum sativum*), bean (*Phaseolus vulgaris*) (Fabaceae), carrot (*Daucus carota*) (Apiaceae), and sugar beet (*Beta vulgaris*) (Chenopodiaceae). Superficially sterilized commercial seed of all tested plant species were placed on PDA slants in 20-cm glass tubes and incubated at 23 to 25°C and a cycle of 12 h of light and 12 h of darkness, until well-developed cotyledons were visible. In each tube, a 5-mm mycelial plug from a 7-day-old culture of a respective isolate was placed on the plantlet roots (mycelial surface face down). Seedlings of each plant species inoculated with sterile PDA served as negative control. The inoculated tubes were randomized and returned to the incubator for additional 7-day incubation, followed by aggressiveness evaluation. Symptom intensity were rated using the following scale, established during this study: 0 = no reaction; 1 = up to 30% of roots affected; 2 = up to 40% of roots affected, 3 = total of 40 to 60% of roots affected, and 4 = roots and entire plantlet necrotic and decayed. The inoculations were performed in five replications, and the entire experiment was conducted twice.

Statistical analysis. The colony diameters on different temperatures and disease severity indexes in the inoculation trials were verified for normality by Colmogorov-Smirnov and Liliefors tests using Graph Pad Software 5.0, and processed by factorial analysis of variance using Statistica 7 (StatSoft). The means were compared by Tukey's test at the $P < 0.05$ significance level.

The disease severity indexes in the inoculation trials, as ordinal qualitatively measured data, were subjected to the nonparametric statistic test of Kruskal-Wallis for each isolate separately. The medians of disease severity were compared using Dunn's multiple comparison test at the $P < 0.05$ significance level.

Results

Disease symptoms and isolates. Cabbage and oilseed rape plants with symptoms of yellowing and wilting, occasionally followed by the whole plant collapsing, were randomly distributed in groups or patches of different sizes in both crops. Disease incidence was estimated at 15 and 20%, respectively. Roots of sampled plants exhibited symptoms of crown and partial root necrosis (Fig. 1A and B). From all collected symptomatic cabbage and oilseed rape plants, only *Rhizoctonia*-like colonies were recovered, all with uniform colony appearance. In total, 12 hyphal-tip isolates (6 from each location) were selected for further study.

Fungal morphology and AG reaction. Regardless of the origin, all isolates exhibited uniform morphological features forming round, fast-growing colonies which were white when young and became salmon pink to orange in color with age (Fig. 1C). After 4 to 7 days, all isolates formed moniloid cells (Fig. 1D) and, after 7 to 10 days, small red to brown superficial or partly immersed sclerotia formed, 0.5 to 1 mm in diameter (Fig. 1E and F). All isolates exhibited typical *Rhizoctonia*-like branching at a right angle, with the presence of slight constriction and septum near the branching. All isolates had multinucleate hyphal cells containing 3 to 10 nuclei per cell. Similarly, all isolates were able to anastomose only with each other and with none of the available tester isolates. The observed morphological features indicated that the Serbian isolates from cabbage and oilseed rape were similar to *Waitea circinata* var. *zeae*.

Temperature requirements. Growth rate of both isolates regardless of the origin was significantly affected by temperature ($P < 0.01$) (Fig. 2). Neither isolate was able to grow at 5, 10, and 40°C, while the fastest growth was recorded at 30°C, as the optimal temperature. The differences in growth rate between isolates were also significant at all studied temperatures ($P < 0.01$).

Molecular identification and phylogenetic analyses. Identification of Serbian *W. circinata* var. *zeae* cabbage and oilseed rape isolates was further supported by BLAST sequence analyses of all four targeted genome regions (ITS, LSU, *RPB2,* and β-*tubulin*). Sequence analyses of the ITS region of 12 Serbian isolates (Table 3) revealed that they share nucleotide similarity of 99.62 to 100% (0- to 2-bp differences). BLAST analysis also revealed nucleotide sequence similarity of 99 to 100% with over 70 sequences of *W. circinata* var. *zeae* from different parts of the world and different host plants. Sequence analyses of the LSU region of two Serbian *Waitea* isolates (299-17 and 300-17, accession numbers MN121346 and MN121344, respectively) revealed that they share nucleotide

Table 1. *Waitea circinata* var. *zeae* isolates characterized in this study

Isolate	Host plant	Morph[a]	Temp[b]	ITS[c]	LSU, *RPB2*, β-*tubulin*[d]	Testing[e]	Range[f]
299-17	Cabbage	+	+	+	+	+	+
299-17-1	Cabbage	+	−	+	−	+	−
299-17-2	Cabbage	+	−	+	−	+	−
299-17-3	Cabbage	+	−	+	−	+	−
299-17-4	Cabbage	+	−	+	−	+	−
299-17-5	Cabbage	+	−	+	−	+	−
300-17	Oilseed rape	+	+	+	+	+	+
300-17-1	Oilseed rape	+	−	+	−	+	−
300-17-2	Oilseed rape	+	−	+	−	+	−
300-17-3	Oilseed rape	+	−	+	−	+	−
300-17-4	Oilseed rape	+	−	+	−	+	−
300-17-5	Oilseed rape	+	−	+	−	+	−

[a] Morphology and anastomosis group pairing.
[b] Temperature requirements.
[c] Internal transcribed spacer (ITS) sequencing and phylogeny.
[d] Large subunit (LSU), *RNA polymerase II* (*RPB2*), and β-*tubulin* sequencing.
[e] Pathogenicity testing.
[f] Aggressiveness and host range.

Table 2. Primers used for the sequencing of *Waitea* spp. and PCR conditions

Loci[a]	Primer name	Sequences 5′–3′	Annealing conditions[b]	Reference
ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA	1 min, 52°C	Gardes and Bruns 1993
	ITS4	TCCTCCGCTTATTGATATGC	…	White et al. 1990
LSU	LROR	GTACCCGCTGAACTTAAGC	45 s (5 cycles), 50°C	Vilgalys and Hester 1990
	LR5	ATCCTGAGGGAAACTTC	…	Vilgalys and Hester 1990
RPB2	BRPB26F	TGGGGYATGGNTTGYCCYGC	45 s, 60°C (5 cycles), then 58 to 54°C (−1°C cycle^{-1})* (5 cycles), then 30 s, 54°C **	Matheny 2005
	BRPB271R	CCCATRGCYTGYTTMCCCAT	…	Reeb et al. 2004
β-*Tub*	B36F	CACCCACTCCCTCGGTGGTG	45 s, 58°C	Thon and Royse 1999
	B12R	CATGAAGAAGTGAAGACGCGGGAA	…	Thon and Royse 1999

[a] ITS = internal transcribed spacer, LSU = large subunit, *RPB2* = *RNA polymerase II*, and β-*Tub* = β-*tubulin*.
[b] Asterisks: * indicates that, in each cycle, the temperature was lowered by 1°C and ** indicates a total of 40 cycles for PCR, whereas all other PCRs were performed with total of 35 cycles.

Table 3. *Waitea* and *Waitea*-related internal transcribed spacer sequences derived from GenBank included in the phylogenetic analysis

Accession number	Isolate	Species[a]	Host (previous crop, family)[b]	Country	Literature
KM065560	CNPAF_0080	*zeae*	Soil (coffee, Rubiaceae)	Brazil	Blanco et al. 2018
KM065553	CNPAF_0056	*zeae*	Soil (soybean, Fabaceae)	Brazil	Blanco et al. 2018
KX468798	CNPAF_0102	*zeae*	Soil (bean, Fabaceae)	Brazil	Blanco et al. 2018
KX468799	CNPAF_0105	*zeae*	Soil (bean, Fabaceae)	Brazil	Blanco et al. 2018
KX468810	CNPAF_0148	*zeae*	Soil (soybean, Fabaceae)	Brazil	Blanco et al. 2018
KX468814	CNPAF_0178	*zeae*	Soil (bean, Fabaceae)	Brazil	Blanco et al. 2018
KC620580	CrT21	*zeae*	Soil (tobacco, Solanaceae)	Turkey	Aydın et al. 2013
KC620581	Yakakent	*zeae*	Soil (tobacco, Solanaceae)	Turkey	Aydın et al. 2013
EU591758	R13	*zeae*	Carrot (Apiaceae)[c]	United States	Ohkura et al. 2009
EU591763	R18	*zeae*	Bean (Fabaceae)	United States	Ohkura et al. 2009
JX073667	HLJ-RZ1	*zeae*	Sugar beet (Amaranthaceae)	China	Zhao et al. 2019
KT428732	GS-39	*circinata*	Sugar beet (Amaranthaceae)	China	Zhao et al. 2019
KJ623715	Hall	*zeae*	Soil (sugar beet, Amaranthaceae)	United States	Webb et al. 2015
AB213571	KAR.BFW	*agrotis*	Kentucky bluegrass (Poaceae)	Japan	Toda et al. 2007
AB213572	TOU.FW	*agrotis*	Kentucky bluegrass (Poaceae)	Japan	Toda et al. 2007
AB213588	Ro36	*oryzae*	Rice (Poaceae)	Japan	de la Cerda et al. 2007
FJ766523	MWC5	*oryzae*	Rice (Poaceae)	Myanmar	Unpublished
AB213581	KT.08.1	*circinata*	Creeping bentgrass (Poaceae)	Japan	Toda et al. 2007
AB213582	HTB.A.1	*circinata*	Zoysiagrass (Poaceae)	Japan	Toda et al. 2007
MK418799	Ce-2	*circinata*	Creeping bentgrass (Poaceae)	Spain	Gomez de Barreda et al. 2019
HM597143	SK.HBA.W1	*prodigus*	Seadwarf (Poaceae)	United States	Kammerer et al. 2011
HM597146	SK.PSA.TM4	*prodigus*	Seadwarf (Poaceae)	United States	Kammerer et al. 2011
KX468819	CNPAF_0191	*zeae*	Soil (onion, Alliaceae)	Brazil	Blanco et al. 2018
HQ270169	YR.185	*zeae*	Corn (Poaceae)	China	Unpublished
KC620583	Hungary	*zeae*	Festuca sp. (Poaceae)	Hungary	Aydın et al. 2013
DQ900594	OCGC 1.1	*zeae*	Meadow fescue (Poaceae)	Japan	de la Cerda et al. 2007
HM597142	SK.820.BG	*zeae*	Bermuda grass (Poaceae)	United States	Kammerer et al. 2011
KC620577	Brazil	*zeae*	Corn (Poaceae)	Brazil	Aydın et al. 2013
JQ350855	296	*zeae*	Sorghum (Poaceae)	Iran	Unpublished
AB213593	M008	*zeae*	Rice (Poaceae)	Japan	Toda et al. 2007
KC620576	M003	*zeae*	Soil (no data)	Turkey	Aydın et al. 2013
MK817577	299-17	*zeae*	Cabbage (Brassicaceae)	Serbia	This study
MN160233	299-17-1	*zeae*	Cabbage (Brassicaceae)	Serbia	This study
MN160232	299-17-2	*zeae*	Cabbage (Brassicaceae)	Serbia	This study
MN160231	299-17-3	*zeae*	Cabbage (Brassicaceae)	Serbia	This study
MN160234	299-17-4	*zeae*	Cabbage (Brassicaceae)	Serbia	This study
MN160240	299-17-5	*zeae*	Cabbage (Brassicaceae)	Serbia	This study
MK817602	300-17	*zeae*	Oilseed rape (Brassicaceae)	Serbia	This study
MN160252	300-17-1	*zeae*	Oilseed rape (Brassicaceae)	Serbia	This study
MN160242	300-17-2	*zeae*	Oilseed rape (Brassicaceae)	Serbia	This study
MN160253	300-17-3	*zeae*	Oilseed rape (Brassicaceae)	Serbia	This study
MN160250	300-17-4	*zeae*	Oilseed rape (Brassicaceae)	Serbia	This study
MN160251	300-17-5	*zeae*	Oilseed rape (Brassicaceae)	Serbia	This study
KP202862	RWB-3	AG1 IB	Rosemary (Lamiaceae)	Korea	Aktaruzzaman et al. 2015

[a] All species are *Waitea circinata* varieties, except for *Rhizoctonia solani* AG1 IB.
[b] Isolates recovered from agricultural soil or plants, with the last cropping history, where relevant, plus family name of the plant in parentheses.
[c] Isolates recovered from natural host plant infection.

similarity of 99.99% (2-bp differences), and 98.4 to 99.2% nucleotide similarities with the four sequences of *W. circinata* var. *oryzae,* because there was no *W. circinata* var. *zeae* available in GenBank. Sequence analyses of the *RPB2* gene of Serbian *Waitea* isolates (299-17 and 300-17, accession numbers MN196475 and MN196476, respectively) revealed that they share similarity of 98.88% (10-bp differences), and the highest nucleotide sequence similarity of 91.12% with the sequence of *W. circinata* var. *zeae* (DQ846899) and one unpublished *Ceratobasidium* sp. sequence (DQ301718), and 90.18% with 14 *W. circinata* var. *zeae* and var. *oryzae* sequences from Brazil (LR606287-300). Sequence analyses of the β-*tubulin* gene of Serbian *Waitea* isolates (299-17 and 300-17, accession numbers MN165454 and MN165455, respectively) revealed that they share 100% identity (0-bp differences), while both shared nucleotide similarity of 96 to 100% with seven sequences of *W. circinata* var. *zeae*, and 93 to 97% nucleotide similarity with two *W. circinata* var. *oryzae* isolates, the only ones available in GenBank.

Phylogenetic neighbor-joining analyses of ITS sequences of 12 Serbian and 31 selected *Waitea* isolates belonging to all five varieties (*W. circinata* var. *zeae, oryzae, circinata, prodigus,* and *agrotis*) resulted in a phylogenetic tree with topology and resolution consistent with previous identification of publicly available isolates (Fig. 3). All *W. circinata* var. *zeae* isolates formed one defined branch, more closely related to *W. circinata* var. *circinata, prodigus,* and *agrotis*, and all were distant from the isolates of *W. circinata* var. *oryzae*. The branch of *W. circinata* var. *zeae* isolates further clustered in two main groups, designated as I and II. All 12 Serbian as well as the vast majority of *Waitea* isolates originating from natural infection of dicotyledonous plants clustered in group I, exhibiting high nucleotide similarity with 0- to 2-bp differences. Monophyletic group II comprised two distinct branches, designated as IIa and IIb, with higher nucleotide diversity (0 to 15 and 0 to 6, respectively). Both branches comprise the isolates originating from monocotyledonous plants, with the exception of sublineage IIa and the isolate from carrot from the United States, which proved to be more distantly related to all other isolates pathogenic for dicotyledonous host plants.

Pathogenicity and host range aggressiveness. Pathogenicity of 12 selected isolates from oilseed rape and cabbage was proven, because they caused visible symptoms of root necrosis on challenged seedlings 7 dpi, thus fulfilling Koch's postulates. All isolates exhibited uniform pathogenicity, causing similar reactions in symptom appearance and intensity on inoculated seedlings. All isolates were reisolated from necrotic roots of all 10 inoculated cabbage and oilseed rape seedlings, thus fulfilling Koch's postulates, while control seedlings remained symptomless.

No significant differences in aggressiveness were observed between two selected Serbian *W. circinata* var. *zeae* isolates which caused mild to medium root necrosis on seedlings of all 14 artificially inoculated plant species 7 dpi. Seedlings of some of the inoculated plants such as sunflower reacted with intensive root necrosis followed by whole-seedling decay. Symptoms were of similar appearance regardless of isolate or host plants. Cabbage and oilseed rape exhibited symptoms similar to natural infections but were more intensive on cabbage regardless of the challenging isolate (Fig. 4). Control seedlings of all inoculated hosts showed no symptoms. Both isolates were recovered from all symptomatic seedlings. Statistical analysis of the ordinal data revealed that symptom development was significantly affected by the host plants ($P < 0.0001$), regardless the isolate used for inoculation. Isolate 299-17 was the least aggressive on cucumber, wheat, and oilseed rape, while 300-17 was the least aggressive on bean, followed by tobacco, wheat, cucumber, maize, and oilseed rape. Isolate 299-17 exhibited the highest virulence on sunflower, while 300-17 was the most aggressive on carrot and sunflower. The highest median value of the disease severity index was recorded on sunflower and the lowest on cucumber (Fig. 4).

Discussion

In this article, to the best of our knowledge, we are describing the first record of *W. circinata* var. *zeae* as the causal agent of root rot of cabbage and oilseed rape. This study documented a previously unknown capability of W. *circinata* var. *zeae* to cause natural infection of two host plants from the Brassicaceae family. Disease incidence on both cabbage and oilseed rape crops in Serbia was estimated at 15 and 20%, respectively, demonstrating the capability of *W. circinata* var. *zeae* to cause substantial yield losses. Numerous AGs of multinucleate *Rhizoctonia* spp. were previously recorded as pathogens of cabbage and different varieties of *Brassica oleracea,* including AG-1, AG-1-IA, AG-1-IB, AG-1-IC, AG-2-1, AG-2-2, AG-2-2-IIIB, AG-2-2-IV AG-4; AG-4-HGI, AG-4-HGII, AG-4HGIII AG-3, AG-5, and AG-7, as well as binucleate *Rhizoctonia* AG-A (Abawi and Martin 1985; Budge et al. 2009; Hua et al. 2014; Ireland et al. 2015; Keinath and Farnham 1997; Kubota and Abiko 1997; Kubota et al. 2009; Kuramae et al. 2003; Misawa and Aoki 2017; Misawa et al. 2015; Ohkura et al. 2009; Pannecoucque et al. 2008; Sayama 2000; Yang et al. 2007). Similarly, multinucleate AG-2-1, AG-2-2, AG-10, AG-4, AG-5, AG-8, AG-9, and AG-10, as well as binucleate *Rhizoctonia* AG-K and *Ceratobasidium* spp., were detected as pathogens of oilseed rape and genotypes of *B. napus* (Broders et al. 2014; Hannukkala et al. 2016; Khangura et al. 1999; Paulitz et al. 2006; Schroeder and Paulitz 2012; Yang et al. 1996; Zhou et al. 2014).

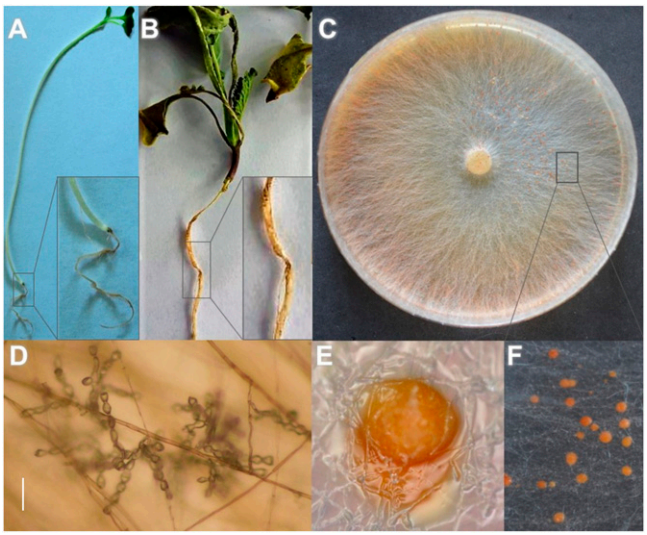

Fig. 1. *Waitea circinata* var. *zeae*. **A,** Root necrosis of naturally infected cabbage plants. **B,** Root necrosis of naturally infected oilseed rape plants. **C,** Colony appearance on potato dextrose agar (PDA) 7 days postinoculation (dpi). **D,** Moniloid cells in colonies 5 dpi (scale bar = 100 μm). **E,** Sclerotia partly immersed in the media. **F,** Red sclerotia on PDA 21 dpi.

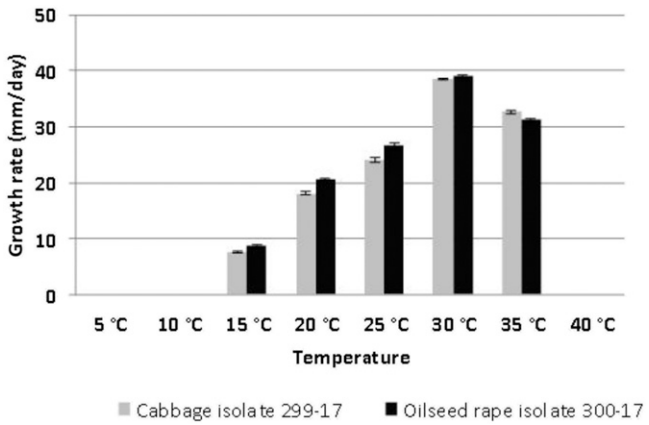

Fig. 2. Growth rate of *Waitea circinata* var. *zeae* cabbage isolate 299-17 and *W. circinata* var. *zeae* oilseed rape isolate 300-17, at temperatures from 5 to 40°C with 5°C intervals.

With its soilborne nature, the occurrence of *W. circinata* var. *zeae* detected in our study on such widely grown and important crops worldwide is of great concern.

Moreover, this is the first record of *W. circinata* var. *zeae* naturally infecting any dicotyledonous host in Europe. Thus far, there are only two reports on the presence of *Waitea* spp. in Europe and both are related to monocotyledons plants. *W. circinata* var. *zeae* was reported on *Lolium perenne* and *Festuca* spp. in Hungary (Vajna and Oros 2005) and, more recently, *W. circinata* var. *circinata* was reported on *Agrostis stolonifera* in Spain (Gómez de Barreda et al. 2019). It seems that, in different parts of the world, *W. circinata* var. *zeae* has adapted to infect dicotyledonous plants. After recent reports on several hosts from the families Fabaceae (Erper et al. 2005, 2011; Ohkura et al. 2009; Sharma-Poudyal et al. 2015;

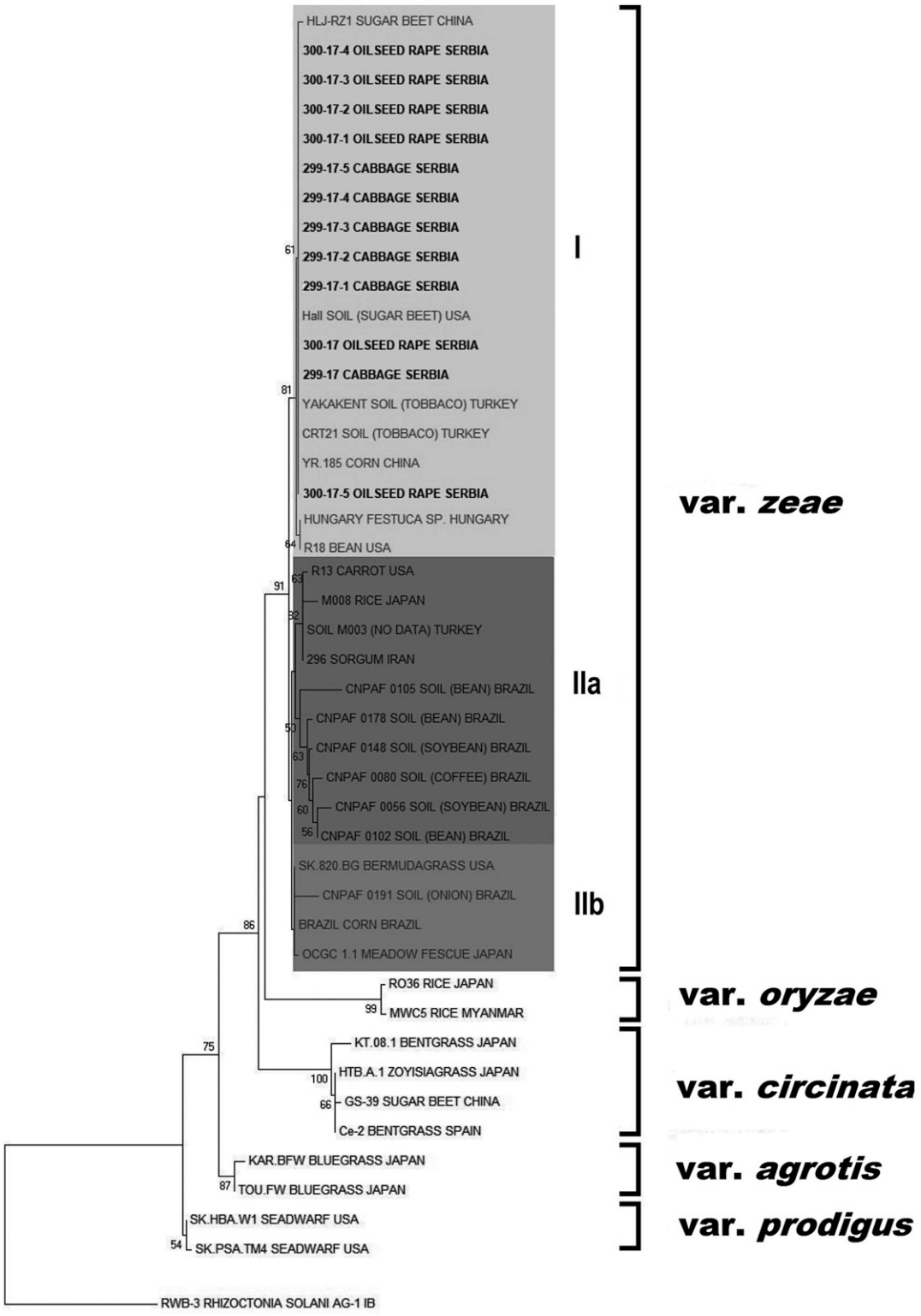

Fig. 3. Neighbor-joining phylogenetic tree of internal transcribed spacer ribosomal DNA sequences of 12 Serbian isolates, 31 reference *Waitea circinata* isolates, and outgroup taxa *Rhizoctonia solani* AG-1 IB. The tree was generated in MEGA 6.0 using Kimura's two-parameter model (Tamura et al. 2013). Bootstrap analyses was performed with 1,000 replicates, and bootstrap values (>50%) are shown next to the relevant branches. Serbian *W. circinata* var. *zeae* isolates appear in bold.

Tewoldemedhin et al. 2015), Apiaceae, and Chenopodiaceae (Kuznia and Windels 1994; Ohkura et al. 2009; Zhao et al. 2019), it appears that we have detected and characterized isolates with a new trait, pathogenicity for two species from the Brassicaceae family. Pathogenicity and host range expansion mechanisms of plant pathogens are a complex phenomenon, not frequently addressed in literature. A recent study showed diversification regarding host range of both *R. solani* AG-1 IA from rice and soybean and *R. oryzae* from rice to *Urochloa* spp. (Chavarro Mesa et al. 2015; Pereira et al. 2017). The prediction of future outbreaks and new host plants of *W. circinata* var. *zeae* should rely on research on host range diversification and evolutionary patterns of adaptation, which requires much more data and a larger number of fully characterized isolates.

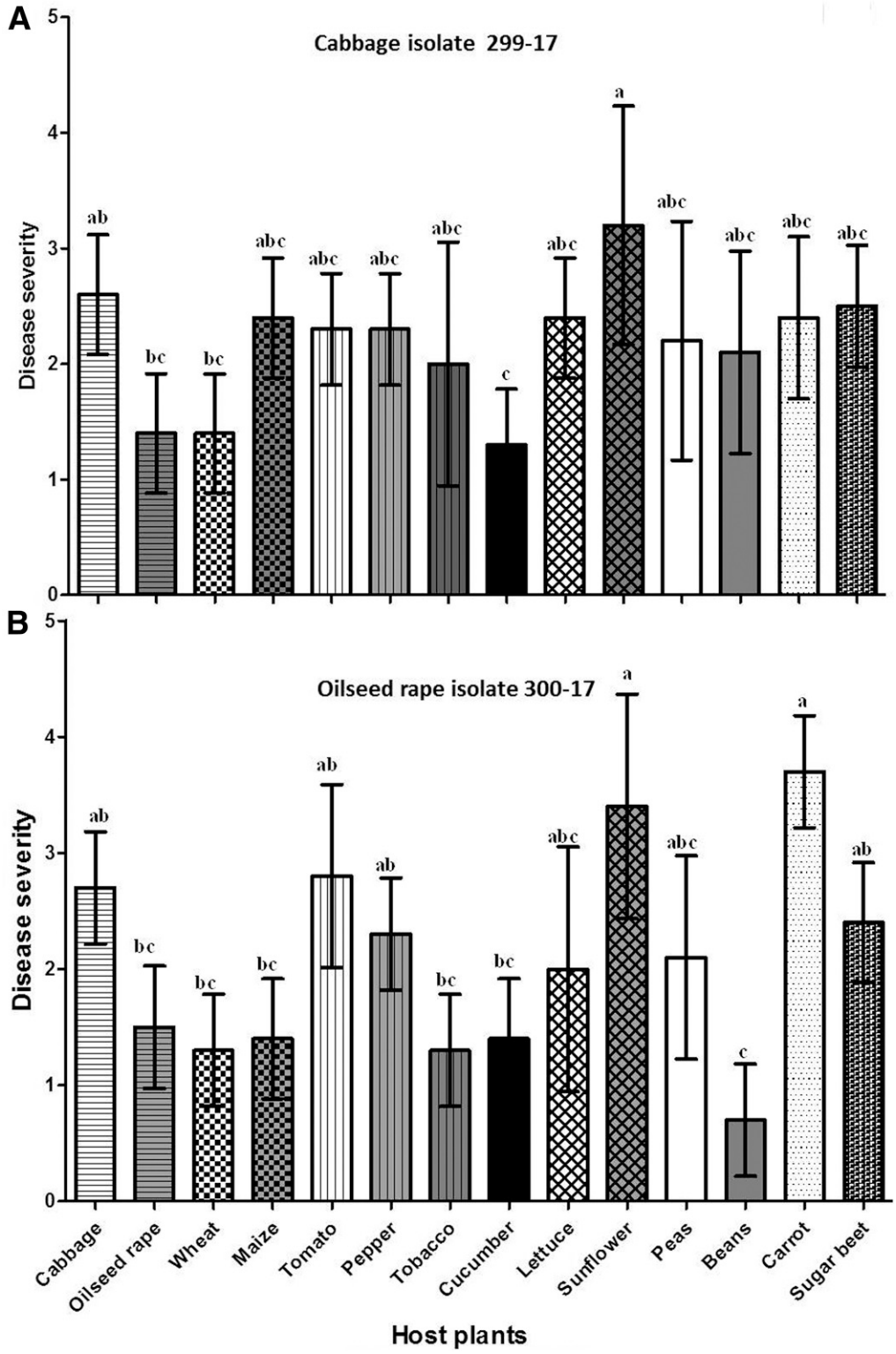

Fig. 4. Root rot severity on different host plants caused by *Waitea circinata* var. *zeae* isolates from **A,** cabbage (299-17) and **B,** oilseed rape (300-17) evaluated with the following scale: 0 = no reaction, 1 = up to 30% of roots affected, 2 = up to 40% of roots affected, 3 = total of 40 to 60% of roots affected, and 4 = roots and entire plantlet necrotic and decayed.

Regardless of the host plant from which they originated, all morphological and ecological features of Serbian *W. circinata* var. *zeae* isolates are uniform and in accordance with previously published data (Erper et al. 2005, 2006; Martin and Lucas 1984; Ploetz et al. 1985; Sumner and Bell 1982). All Serbian isolates were able to anastomose only with each other, implying that all can be attributed to the same AG, as was previously published for different varieties of *W. circinata* (Burpee and Martin 1992). A particularly interesting feature of *W. circinata* var. *zeae* is a high temperature optimum of 30 to 32°C (Erper et al. 2005; Leiner and Carling 1994; Martin and Lucas 1984; Tomaso-Peterson and Trevathan 2007). Regardless of slight differences, both Serbian isolates exhibited the fastest growth rate at 30°C. This trait could be a comparative advantage of *W. circinata* var. *zeae* contributing to recent new outbreaks. Climate change and warm weather in Europe and worldwide, coupled with a frequently practiced narrow crop rotation or complete absence of rotation, could be favorable for the occurrence of *W. circinata* var. *zeae* in new geographic areas and on new host plants.

Several phylogenetic analyses discussed relationships among *Rhizoctonia* spp. with limited or no *Waitea* isolates included (Mercado Cárdenas et al. 2015; Ohkura et al. 2009; Sharon et al. 2008; Zhao et al. 2019). In addition, several studies explored the boundaries and relationships among and within different *W. circinata* varieties (Aydın et al. 2013; Gurkanli et al. 2016; Kammerer et al. 2011; Toda et al. 2007). Direct sequencing of four targeted genome regions (ITS, LSU, *RPB2*, and β-*tubulin*) proved that only the ITS region is reliable for variety-level identification. Due to the small number of available sequences in GenBank, only species-level identification is achievable by using direct sequences of the LSU, *RPB2*, and β-*tubulin* genomic regions. Our phylogenetic analyses aimed at inferring possible evolutionary relationships between isolates of *W. circinata* var. *zeae,* particularly originating from dicotyledonous host plants, as well as between the isolates of similar geographic origin. We have included all of the available *W. circinata* var. *zeae* strains associated with dicotyledonous plants, either from natural infections or baited from agricultural soil with a previous dicotyledonous crop, along with the strains from monocotyledonous hosts representing the main geographical area with *W. circinata* var. *zeae* presence. Our results support the existence of the previously published two main groups, and the distinction of two sublineages within the second group (Aydın et al. 2013; Gurkanli et al. 2016). With the exception of the U.S. carrot isolate, group I of the *W. circinata* var. *zeae* isolates comprised all of the isolates originating from dicotyledonous plants as well as the oilseed rape and cabbage isolates from Serbia. The geographic origin of isolates and their grouping supported the previously published hypothesis of possible routes of dissemination. Aydın et al. (2013) argued that a possible dissemination route of *W. circinata* var. *zeae* isolates originated from the Americas, via the Far East and Asia to Turkey and Eastern Europe, which is supported by our results. Thus far, in Europe, *W. circinata* var. *zeae* has been reported only in Hungary, on monocotyledonous *L. perenne* and *Festuca* spp. (Vajna and Oros 2005), and it is likely that the introduction in Serbia is the result of the same dissemination route. Recently, another variety of *W. circinata* has been detected in Europe, *W. circinata* var. *circinata* in Spain (Gómez de Barreda et al. 2019), but that is most probably the result of a separate introduction.

The 12 *W. circinata* var. *zeae* isolates, 6 from oilseed rape and 6 from cabbage, expressed uniform pathogenicity in symptom appearance and intensity while fulfilling Koch's postulates. For that reason, one isolate originating from each crop was selected to explore their experimental host range and aggressiveness, a feature rarely studied in literature. Aggressiveness of Serbian *W. circinata* var. *zeae* isolates on a range of experimental host plants implies their capability to infect a broader spectrum of young host plants, particularly during germinating and emergence, than previously tested and published (Bittsanszky et al. 2015; Erper et al. 2005, 2006, 2011; Leiner and Carling 1994; Martin and Lucas 1984; Ohkura et al. 2009; Oros 2015; Oros et al. 2013; Sumner and Bell 1982; Tewoldemedhin et al. 2015; Toda et al. 2007; Tomaso-Peterson and Trevathan 2007; Zhao et al. 2019). Serbian isolates did not express differences in their aggressiveness, and exhibited the highest aggressiveness on sunflower (Asteraceae), which is not listed as a natural host plant of *W. circinata* var. *zeae*. Although additional glasshouse and field pathogenicity trials are needed for detailed insight into the pathogenicity of *W. circinata* var. *zeae*, these results suggest that the host range of this pathogen is broader than previously thought.

The results of our study contributed to the characterization of *Waitea* spp. and *W. circinata* var. *zeae*, a greatly understudied group of soilborne pathogens. With regards to pathogenicity, *W. circinata* var. *zeae* is extremely diverse, comprising nonpathogenic isolates recommended for biological control (Webb et al. 2015) but also isolates pathogenic for a broad spectrum of cereal crops and grasses, as well as several important vegetable and agricultural crops (Erper et al. 2005, 2011; Kuznia and Windels 1994; Ohkura et al. 2009; Sharma-Poudyal et al. 2015; Tewoldemedhin et al. 2015, Zhao et al. 2019). In regards to widening the host range and occurring in new geographic areas, future studies of *W. circinata* var. *zeae* should clarify several important epidemiological aspects, including host range, long-term preservation, and, above all, means of dispersal.

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