Molecular identification and characterization of binucleate *Rhizoctonia* spp. associated with black root rot of strawberry in Serbia

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SUMMARY

Strawberry production is a popular, fast-growing agricultural business in Serbia. Its cultivar selection has been changing fast, following market demands. One of the limiting factors of strawberry production worldwide is black root rot, primarily caused by binucleate *Rhizoctonia*. Recently, outbreaks of black root rot of strawberry have occurred in Serbia and the estimated disease incidence was up to 30%. Isolates of binucleate *Rhizoctonia* AG-A were recovered from symptomatic strawberry plants, and characterized on the bases of morphological, molecular and pathogenic features. Despite their uniform morphological characteristics, the isolates demonstrated genetic variability within ITS rDNA, grouping into three different phylogenetic sub-clusters which comprise AG-A isolates originating from Italy, Israel, Japan and the USA. The binucleate *Rhizoctonia* AG-A from Serbia exhibited uniform virulence on strawberry after inoculation of daughter plants and detached leaf petioles, as well as on seedlings of bean, carrot and sunflower, while they were non-pathogenic to wheat, maize, tomato, pepper, tobacco, cucumber, lettuce, peas, cabbage, rapeseed and sugar beet.

Keywords: *Rhizoctonia*; Hyphal anastomosis*;* Binucleate AG-A; ITS rDNA sequence analyses; Pathogenicity; Strawberry

INTRODUCTION

Strawberry (*Fragaria* x *ananassa* Duchesne ex Rozier, family Rosaceae) is one of the most economically important berry crops worldwide. In 2016, strawberry was grown on over 400,000 ha and total world production reached almost 9.2 million tons (http://www.fao.org/faostat). In Serbia, strawberry is also among the most important berry crops, and it is grown on more than 7,800 ha (Nikolić & Milivojević, 2015). Occasionally, black root rot disease occurs as a limiting factor of strawberry production worldwide, which is primarily caused by the binucleate *Rhizoctonia* spp. and several other non-lethal pathogens, such as *Pythium* spp., *Cylindrocarpon* sp., *Fusarium* spp.

and nematodes (Martin, 1988; Botha et al., 2003; Sharon et al., 2007; Manici & Bonora, 2007; Fang et al., 2013). Annual yield reductions due to black root rot of 30% or higher have already been reported (Botha et al., 2003). In several production areas, commonly practiced soil fumigation has successfully reduced the economic impact of soil-borne pathogens, including those associated with black root rot of strawberry. However, proscription of soil fumigation with methyl-bromide has remarkably altered production practices. As a consequence, black root rot of strawberry became significant in several production areas, including the USA (Martin, 2000), South Africa (Botha et al., 2003), Italy (Manici & Bonora, 2007), Israel (Sharon et al., 2007), Japan (Matsumoto & Yoshida, 2006), and Australia (Fang et al., 2011).

Rhizoctonia spp. comprise worldwide distributed, soil-borne fungi that are pathogenic to a range of important cultivated plants. *Rhizoctonia* spp. are classified into three groups based on their cell nuclear condition: multinucleate *Rhizoctonia* (teleomorphs: *Thanatephorus* and *Waitea*), binucleate *Rhizoctonia* (teleomorphs: *Ceratobasidium* and *Tulasnella*) and uninucleate *Rhizoctonia* (teleomorph: *Ceratobasidium*) (Ogoshi, 1987). Based on hyphal anastomosis reactions, multinucleate *Rhizoctonia* are further divided into 13 anastomosis groups (AG) (AG-1-13) and binucleate *Rhizoctonia* into 23 anastomosis groups (AG-A to AG-W) (Sharon et al., 2008; Yang et al., 2015). The binucleate *Rhizoctonia* spp. as the primary agent of black root rot is commonly referred to as *Rhizoctonia fragariae* (Martin, 1988; Botha et al., 2003; Sharon et al., 2007; Fang et al., 2013). *R. fragariae* was first described as the causal agent of strawberry planting degeneration in Canada (Husain & McKeen, 1963). Further steps of conventional and molecular characterization highlighted three anastomosis groups: AG-A, AG-G, and AG-I to be the most commonly associated with *R. fragariae* (Ogoshi et al., 1979; Ogoshi et al., 1984; Martin, 2000; Matsumoto & Yoshida, 2006; Sharon et al., 2007; Fang et al., 2013; Botha et al., 2003). However, in some strawberry growing regions, binucleate AG-H, AG-C, AG-F, AG-K, AG-B, and multinucleate *Rhizoctonia solani* AG-4HGI, AG-4HGIII and AG-6 have also been included in the disease complex (Botha et al., 2003; Sharon et al., 2007; Manici & Bonora, 2007; Fang et al., 2013).

Data on the presence, distribution and significance of *Rhizoctonia* spp. in Serbia are limited, and they mainly refer to the binucleate *Rhizoctonia* on strawberry (Vico, 1994) and multinucleate *Rhizoctonia* on sugar beet (Stojšin et al., 2006; Vico et al., 2006). The only available characterization of *Rhizoctonia* isolates pathogenic to

strawberry in Serbia was based on conventional AG pairing (Vico, 1994) and serology with polyclonal antisera (Vico et al., 2002), and the isolates were identified as the binucleate *Rhizoctonia* spp. AG-A and AG-I. Over the recent years, low to medium incidence of black root rot disease has occurred in several strawberry orchards in Serbia, and *Rhizoctonia* spp.-like isolates were invariably recovered from the diseased plants. The main objectives of this study were therefore: (i) to characterize the selected isolates based on morphological features, (ii) to test their pathogenicity on strawberry and selected host plants, and (iii) to determine their taxonomic position based on AG pairing and sequencing of its ITS region (ITS1, 5.8S rDNA and ITS2 regions).

MATERIAL AND METHODS

Fungal isolates

Isolates of *Rhizoctonia* were collected from four separate outbreaks of black root rot of strawberry in 2013-2018. Samples consisting of symptomatic strawberry plants cv. 'Camarosa', were collected in the vicinity of Subotica in 2013, while samples of strawberry cvs. 'Asia' and 'Senga-Sengana' were collected in the vicinity of Belgrade and Novi Sad, respectively, during 2016, and samples of strawberry cv. 'Roxana' in Kruševac environs in 2018. Up to 15 samples were randomly collected on each location, sealed in plastic bags, stored at 5°C and processed within 24-48 h. Tissue fragments from the contact area between necrotic and healthy parts of roots and leaves were washed with tap water for 2 h, surface sterilized for 2 min with 50% commercial bleach (2 % sodium hypochlorite), placed on the potato dextrose agar (PDA; 200 g potato, 20 g dextrose, 17 g agar and 1 liter of distilled H_2O) and incubated at 24°C for 3-5 days. *Rhizoctonia*-like colonies were then transferred onto fresh PDA and water agar (WA, 17 g agar and 1 liter of distilled H_2O) to obtain hyphal tip isolates. The isolates were maintained on PDA slants at 4°C, in the Collection of Fungi of the Department of Phytopathology, Institute of Phytomedicine, University of Belgrade - Faculty of Agriculture.

Morphological identification

Rhizoctonia isolates were identified based on the hyphal branching pattern and characterized on the bases of colony appearance, growth rate, and the number of nuclei in hyphae. Hyphal branching pattern was

examined directly by placing fragments of mycelia in a drop of sterile water and on a glass slide, and observing them under a compound microscope (Olympus CX41). Colony appearance was assessed in 7 days old cultures grown on PDA at 24ºC in darkness, while growth rate was determined by measuring two perpendicular colony diameters in five cultures per isolate, and calculating an average for each isolate. The obtained data were square root transformed and subjected to one-way ANOVA at the 5% probability (Sokal & Rohlf, 1995). Means were separated by Duncan's multiple range test.

The number of nuclei within hyphal cells was determined by a modified clean slide technique (Kronland & Stranghellini, 1988) after staining with aniline blue in lactophenol or safranin O (Herr, 1979). Three positions were treated with wetting solution (0.1ml Tween 20, 0.1ml of 85% lactic acid in 100ml distilled water) in each 3 days old culture, and a treatment with one of the dyes followed. Cover slips were placed over the stained area of colonies and they were allowed to incubate for 10 min. The dye was removed by placing a paper towel on the one side of the cover slip and repeatedly adding a drop of water on the opposite end. The slide was directly viewed using a dark field microscopy on the compound microscope Olympus CX41. The nuclei in 20 cells of young hypha per isolate were counted to confirm the nuclear status of each isolate.

AG determination using conventional methods

Anastomosis reaction tests were performed by pairing the strawberry *Rhizoctonia* isolates with each other, and with the available tester isolates of binucleate AG-D (isolate R13-1) and AG-G (isolate 140-16 from tomato, identified by ITS sequencing), and multinucleate AG-1-1C (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 (kindly provided by Dr James Woodhall, University of Idaho, USA, and Dr Véronique Edel-Herman, INRA France). Testing for AG was done by a modified clean slide technique (Kronland & Stranghellini, 1988, Martin, 2000). Agar fragments with mycelium from the advancing edge of a colony of the unknown *Rhizoctonia* isolates were transferred to a glass slide and paired with a colony of a known tester isolate. Slides were observed regularly and when the hyphae came into contact, the area was covered with an 18-mm coverslip, and the zone of contact was examined, using phase contrast microscopy of the compound Olympus CX41 microscope, for the presence of anastomosis between the different isolates.

The anastomosis reactions were classified as: frequent (more than five fusions observed), weak to intermediate (up to 5 fusions observed) or incompatible (no fusions observed) (Manici & Bonora, 2007). The contact zones in incompatible or weak pairings were reexamined several times.

DNA amplification and sequencing

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, from 100 mg of dry mycelium sampled from 5-7 days old cultures grown on potato dextrose broth (PDB). ITS (Nuclear ribosomal internal transcribed spacer region) rDNA was amplified using the primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). Amplification reactions were performed in a total reaction volume of 25 µl, consisting of 12.5 µl 2 X PCR Master mix (Fermentas, Lithuania), 9 µl RNase-free water, 2.5 µl of both forward and reverse primers (100 pmol/µl, Metabion International, Deutschland) and 1 µl template DNA. Amplification conditions were as follows: initial denaturation step of 2 min at 95°C, followed by 35 cycles of 35 s at 94°C, 1 min at 52°C and 2 min at 72°C, with final extension period of 10 min at 72°C. PCR products were stained in ethidium bromide, analyzed by 1% agarose gel electrophoresis and visualized using a UV transilluminator. The PCR products were sequenced in both directions in an automated sequencer (ABI 3730XL Automatic Sequencer Macrogen Inc., Korea), using the same primers that were used for amplification. Consensus sequences were computed using the ClustalW (Thompson et al., 1994), integrated in MEGA6 software (Tamura et al., 2013), and deposited in the GenBank (http://www.ncbi. nlm.nih.gov). All generated sequences were compared with each other by calculating nucleotide (nt) identities, as well as with previously deposited *Rhizoctonia* spp. isolates available in the GenBank, using the similarity search tool BLAST.

Phylogenetic analyses

The newly generated ITS sequences from four Serbian *Rhizoctonia* isolates were analyzed with eight previously characterized isolates of the AG types of binucleate *Rhizoctonia* sequences, as well as with 16 strawberry isolates from Italy retrieved from the GenBank (Manici & Bonora, 2007) (Table 1). A phylogenetic tree was inferred using the neighbour-joining method (Saitou & Nei, 1987) implemented in MEGA 6.0 software

(Tamura et al., 2013). Distances in 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 on strawberry and additional experimental plants

Rhizoctonia isolates were tested for pathogenicity in three experiments: on stolon-propagated strawberry daughter plants, on detached strawberry leaf petioles and on seedlings of 14 plant species from eight families. After incubation, the pathogen was re-isolated from all symptomatic plants, using the same method as for isolation, and compared with the respective challenging *Rhizoctonia* isolates.

Stolon-propagated disease-free strawberry daughter plants (cv. 'Senga-Sengana') were obtained from a strawberry production nursery. Artificial inoculations were performed using a modified method described by Manici and Bonora (2007). Pots (12.5 cm in diameter and 9 cm high) were half filled with sterilized commercial substrate, onto which 9 cm disks of 7 days old *Rhizoctonia* colony were placed, filled with an additional 3 cm layer of substrate and one strawberry plant was transplanted into each pot. Six plants were inoculated with the selected isolates, while plants transplanted in pots with sterile PDA were used as negative control. Plants were maintained in greenhouse and the presence of symptoms was assessed 30 days post inoculation.

Pathogenicity of *Rhizoctonia* isolates was also tested on detached leaf petioles of strawberry (cv. 'Senga-Sengana') of the same size and age. Petioles were freshly collected, shortened to 20 mm length, superficially sterilized for 30 s in 50% commercial bleach (2 % sodium hypochlorite), and inclined upright into 7 days old cultures of four *Rhizoctonia* isolates grown on PDA at 23°C in darkness. Eight petioles were inoculated per isolate, while petioles inclined in the sterile PDA

Table 1. Isolates of binucleate *Rhyzoctonia* used in phylogenetic analysis

Isolate	AG	Host plant	Geographic Origin	Accession number
R29[a]	AG-A	Strawberry	Italy	AY927331
R31[a]	AG-A	Strawberry	Italy	AY927333
R36[a]	AG-A	Strawberry	Italy	AY927335
R38[a]	AG-A	Strawberry	Italy	AY927337
R39[a]	AG-A	Strawberry	Italy	AY927338
R40[a]	AG-A	Strawberry	Italy	AY927339
R55[a]	AG-A	Strawberry	Italy	AY927347
R59[a]	AG-A	Strawberry	Italy	AY927350
R60[a]	AG-A	Strawberry	Italy	AY927351
R63[a]	AG-A	Strawberry	Italy	AY927352
R70[a]	AG-A	Strawberry	Italy	AY927356
R74[a]	AG-A	Strawberry	Italy	AY927358
R75[a]	AG-A	Strawberry	Italy	AY927359
R77[a]	AG-A	Strawberry	Italy	AY927361
R78[a]	AG-A	Strawberry	Italy	AY927362
R79[a]	AG-A	Strawberry	Italy	AY927363
C-662[b]	AG-A	Soil	Japan	AF354092
Str22[c]	AG-A	Strawberry	Israel	DQ102423
Str36[b]	AG-F	Strawberry	Israel	DQ102435
SIR1[c]	AG-F	Sweet potato	Japan	AF354085
Gm1[c]	AG-G	Strawberry	USA	DQ102395
Str14[c]	AG-G	Strawberry	Israel	DQ102402
Ibs 1[c]	AG-I	Soil	Israel	DQ102442
Im1[c]	AG-I	Strawberry	USA	DQ102443
101-16[d]	AG-A	Strawberry	Serbia	MH517393
107-13[d]	AG-A	Strawberry	Serbia	MH517394
293-16[d]	AG-A	Strawberry	Serbia	MH517395
JK4[d]	AG-A	Strawberry	Serbia	MH517396

(^aManici & Bonora, 2007; ^bGonzales et al., 2001; ^cSharon et al., 2006; ^dthis study)

served as negative control. Inoculated leaf petioles were incubated in a moist chamber and the virulence of the isolates was estimated 7 days post inoculation based on necrosis length. The obtained raw data were square root transformed and subjected to one-way ANOVA at the 5% probability (Sokal & Rohlf, 1995).

The seedlings of wheat and maize (Poaceae), tomato, pepper and tobacco (Solanaceae), cucumber (Cucurbitaceae), lettuce and sunflower (Asteraceae), peas and beans (Fabaceae), cabbage and rapeseed (Brassicaceae), carrot (Apiaceae) and sugar beet (Chenopodiaceae) were artificially inoculated with three *Rhizoctonia* isolates (107-13, 101-16 and 293-16). Superficially sterilized commercial seeds of each species were placed on PDA slants in 20 cm glass tubes and incubated at 23-25°C and in a cycle of 12h light/12h darkness. When well-developed cotyledons were observed, mycelial plugs (5 mm in diameter) from 7 days old cultures of three *Rhizoctonia* isolates were placed on the roots in each tube (mycelial surface face down). Seedlings of each plant species inoculated with sterile PDA served as negative control. The virulence of the isolates was evaluated 7 days post inoculation based on

symptoms observed: (-) no reaction; (+) weak virulence, mild and partial necrosis of main and branch roots; and (++) strong virulence, necrosis of roots, mesocotyl or entire seedling. Each isolate was inoculated onto five seedlings, and the experiment was repeated twice.

RESULTS

Disease symptoms and identification

In all four outbreaks of strawberry black root rot, typical symptoms were observed, commonly distributed in patches and spreading over the rows in orchards. The symptoms included root rot and deterioration (Figure 1A), partial root necrosis (Figure 1B) and partial or complete leaf necrosis (Figure 1C), followed by plant death. Disease incidence was slightly different in each locality, and it was estimated to reach up to 30%. From each of the four locations, 12-15 isolates were recovered from symptomatic strawberry plants, all with uniform morphological features, and four representative isolates were selected for further studies (Table 2).
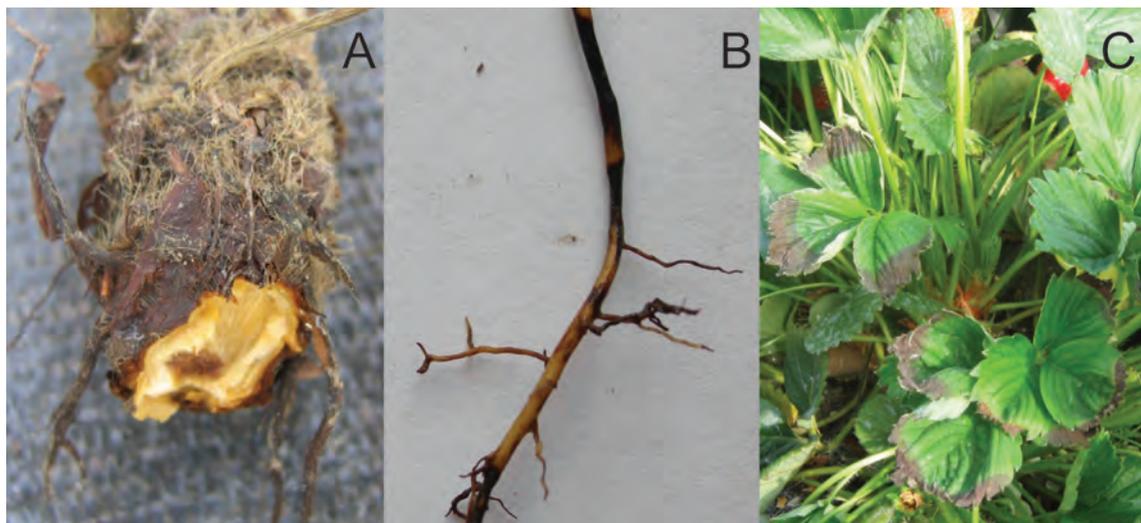

Figure 1. Symptoms on strawberry caused by binucleate *Rhizoctonia* AG-A: Root rot (A); partial root necrosis (B); partial leaf necrosis (C)

Table 2. *Rhizoctonia* isolates from strawberry in Serbia characterized in this study

Isolate	Year of isolation	Location	Cultivar	Isolated from
107-13	2013	Subotica	'Camarosa'	roots
101-16	2016	Belgrade	'Asia'	roots
293-16	2016	Novi Sad	'Senga-Sengana'	leaves
JK4	2018	Kruševac	'Roxana'	roots

Morphological features of the selected isolates were uniform (Table 3). The isolates formed fast growing, white colonies with abundant aerial mycelia. Growth range was from 11.2 mm (isolate 107-13) to 13.2 mm (isolate 101-16), i.e. 12.25 mm on average. Differences among the isolates in their average growth rate were statistically significant (F=49.3; p<0.01). On colony surface, tufts of moniloid cells scattered in discrete concentric rings were visible 7 days post inoculation (Figure 2A). The first typical moniloid cells were detected as soon as 72 h post inoculation (Figure 2B), while none of the isolates formed sclerotia. The hyphae of all isolates were binucleate (Figure 2C) with a typical branching pattern (Figure 2D).
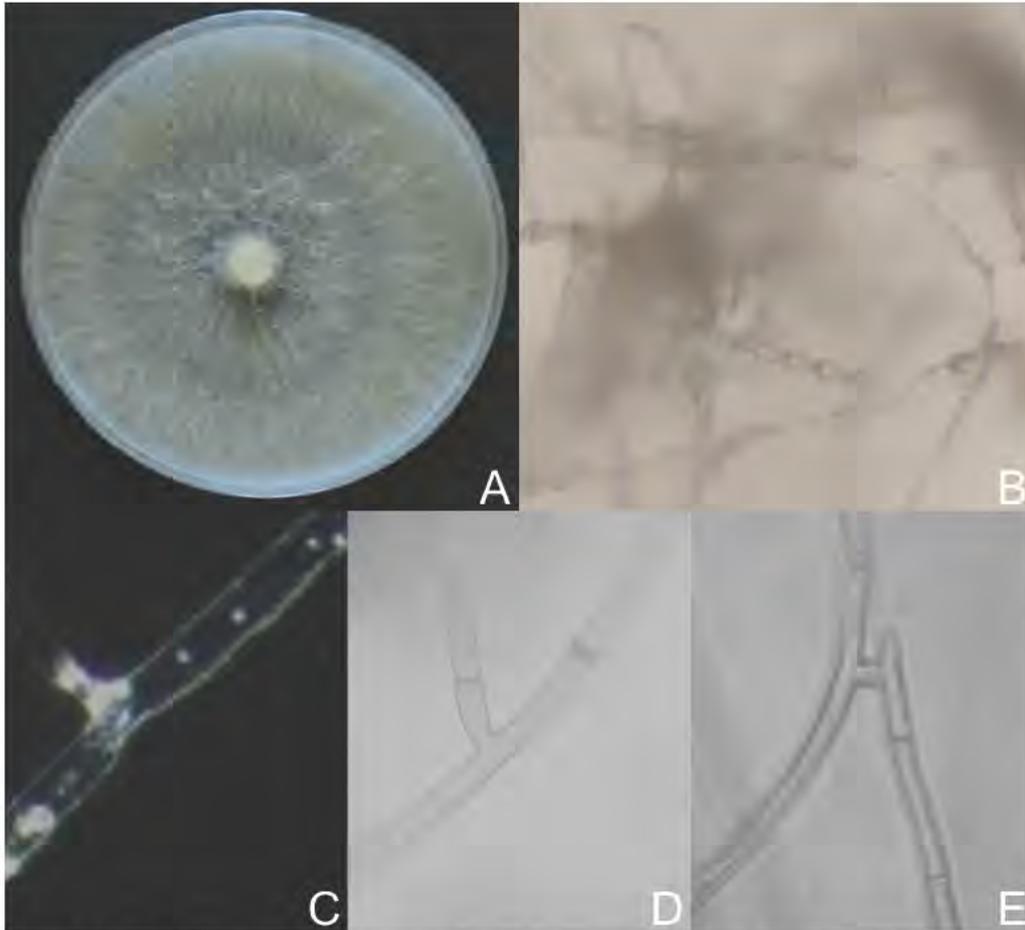
Figure 2. Morphology of strawberry isolates of binucleate *Rhizoctonia* AG-A: Colonies with abundant aerial mycelia and tufts of moniloid cells (A); moniloid cells (B); binucleate hyphae (C); branching pattern (D); hyphal anastomosis (E)

Table 3. Summary information on binucleate *Rhizoctonia* isolates from strawberry in Serbia

Isolate	Colony color	Growth rate	Nuclear condition	Moniliod cells presence	AG type	Pathogenicity on strawberry	
						Daughter plants	Petioles
107-13	white	11.2±0.3 b [a]	binucleate	+	AG-A [b]	+ [c]	16.8±4.8 [d]
101-16	white	13.2±0.7 a	binucleate	+	AG-A	+	14.0±5.7
293-16	white	11.5±0.3 b	binucleate	+	AG-A	+	15.8±3.6
JK4	white	13.0±0.1 a	binucleate	+	AG-A	+	13.6±4.2

[a] Average growth rate (MS±SD) in mm/day - the same letter indicates the difference is not significant; [b] AG type determined by conventional pairing with reference isolates and ITS sequencing; [c] Presence of symptoms of root rot; [d] Average length (MS±SD) of necrotic tissue in mm

Hyphal anastomosis was observed at low to intermediate frequencies only after the pairing of Serbian isolates to each other (Figure 2E). The selected *Rhizoctonia* isolates were not able to form hyphal fusions after pairing to any of the available AG tester isolates of either binucleate or multinucleate *Rhizoctonia* isolates.

Molecular identification and phylogenetic analyses

Sequence analyses of the ITS region of Serbian *Rhizoctonia* isolates revealed that they share the similarity of 97.2-99.2% (up to 10 bp differences), while BLAST analysis revealed the highest nt sequence homology of 99-100% (100% query coverage) with over 70 sequences of binucleate *Rhizoctonia* AG-A from different parts of the world and different host plants.

Neighbor-joining analyses of ITS sequences of 28 binucleate *Rhizoctonia* of AG-A, AG-G, AG-F and AG-I isolates, resulted in a phylogenetic tree with topology and resolution in consistence with previous identification of publicly available isolates (Figure 3). All AG-A isolates formed one well-supported cluster, comprising all four Serbian isolates, as well as the isolates from Italy, Japan, and Israel. Within the AG-A cluster,
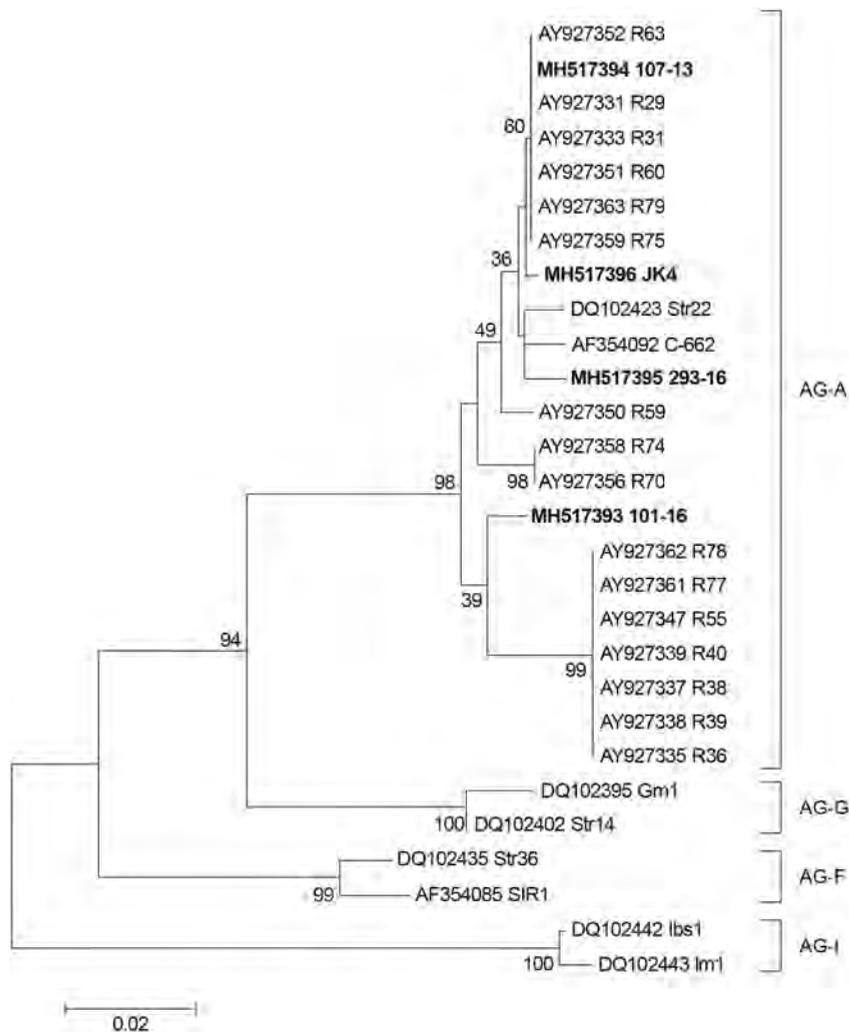
Figure 3. Neighbour-joining phylogenetic tree inferred from ITS rDNA, sequences of 4 Serbian and 24 binucleate *Rhizoctonia* of AG-A, AG-G, AG-F and AG-I isolates originating from Italy, Israel, Japan and the USA. The phylogram was generated with MEGA 6 using Kimura's two-parameter. Bootstrap analysis was performed with 1,000 replicates and bootstrap values (>35%) are shown next to relevant branches. The Serbian binucleate *Rhizoctonia* AG-A isolates are bolded

four additional sub-clusters were defined with medium to strong bootstrap support and the Serbian isolates clustered differently, in three separate branches, indicating a genetic variability.

Pathogenicity

All four Serbian *Rhizoctonia* isolates proved to be pathogenic, causing mild to medium necrosis 30 days post inoculation on the roots of all inoculated strawberry daughter plants. Although mild, the symptoms were consistent with those of natural infection. Control plants showed no tissue discoloration and symptoms. Successful re-isolations from all symptomatic plants complied with Koch' postulates.

First symptoms of black necrosis on detached strawberry leaf petioles were visible 4 days post inoculation, while prominent black necrotic lesions of different lengths were caused by all four isolates, 7 days post inoculation. No necrosis was visible on control leaf petioles inclined into sterile PDA. The isolates 107-13 and 293-16 exhibited slightly higher virulence with average necrosis length of 16.8 and 15.8 mm, respectively (Table 3). However, the observed differences in virulence among the isolates were not statistically significant (F=0.8; p=0.504).

The isolates 107-13, 101-16 and 293-16 exhibited a narrow host range and uniform, medium to low virulence, causing infection of carrot, beans and sunflower, and not of wheat, maize, tomato, pepper, tobacco, cucumber, lettuce, peas, cabbage, rapeseed or sugar beet. Mild symptoms in the form of minute necrotic spots and partial necrosis on main and branch roots of all inoculated seedlings of all three host plants were visible 7 days post inoculation. No symptoms were visible on the seedlings of other inoculated plants nor any of the control seedlings.

DISCUSSION

Considering the importance of strawberry production in Serbia, the soil-borne nature of *Rhizoctonia* and a large-scale international trade and import of strawberry planting material, it is of utmost importance to identify and characterize *Rhizoctonia* isolates that are pathogenic for strawberry in Serbia. The only available data on *Rhizoctonia* causing black root rot on strawberry in Serbia date back in the mid-1990s (Vico, 1994), and they were obtained by morphological, serological and conventional AG pairing methods. Binucleate *Rhizoctonia* spp. AG-A and AG-I were detected. As strawberry cultivar composition has been changing rapidly in our country

over the last 20 or more years (Nikolić & Milivojević, 2015), a data update on *Rhizoctonia* is needed. In addition, data on *Rhizoctonia* pathogenic for strawberry in Europe are limited as well, and the only available research so far was conducted in Italy (Manici & Bonora, 2007).

In four recent outbreaks of strawberry black root rot in Serbia, only binucleate *Rhizoctonia* AG-A was detected, causing typical symptoms. Binucleate *Rhizoctonia* AG-A is the most frequently isolated *Rhizoctonia* in the majority of strawberry producing areas in the world. Although early research in the USA had ranked AG-A as the second prevalent AG (detected in 7.9-26.1% of the samples collected in different years and areas) (Martin, 1988), a more recent survey in California has confirmed AG-A in 68% of symptomatic strawberry samples (Martin, 2000), as well as 69% samples in South Africa (Botha et al., 2003), 68% samples in Italy (Manici & Bonora, 2007), and 71.66% samples in Israel (Sharon et al., 2007).

Uniform morphological features of strawberry AG-A isolates, consistent with all Serbian isolates, has already been described (Botha et al., 2003; Manici & Bonora, 2007; Unal et al., 2014). Only Li et al. (2011) reported a morphological diversity of binucleate *Rhizoctonia* AG-A, describing three morphotypes on the bases of colony morphology. Compared to the description, Serbian isolates resemble type II, defined by developing abundant aerial mycelia, and no sclerotia. In our research, the presence of hyphal fusions was found only in pairings between the Serbian isolates. Our isolates failed to form anastomoses with any of the available AG tester isolates, which, coupled with colony morphology, implied their designation as AG-A. This was further confirmed by ITS sequencing. Conventional pairing for AG determination is difficult and time consuming and sometimes cannot be performed due to incompatibility of certain isolates, while at the same time ITS sequencing has been shown to be a useful tool for overcoming these difficulties (Martin, 2000; Manici & Bonora, 2007; Sharon et al., 2007, 2008; Li et al., 2011; Fang et al. 2013).

The Serbian *Rhizoctonia* AG-A isolates exhibited low variability within ITS region and shared sequence similarity of 97.2-99.2%, which is comparable to the AG-A populations in Italy and Australia, where the isolates shared 95-100% and 94.4-100% similarity, respectively (Manici & Bonora, 2007; Fang et al., 2013). The Italian AG-A population was characterized by reconstruction of a neighbor-joining phylogenetic tree based on ITS sequences of binucleate *Rhizoctonia* of AG-A, AG-G, AG-F and AG-I. Intra AG variability of the Italian AG-A isolates showed their regrouping into four sub-clusters and that is one of rare characterizations of the isolates within the AG-A (Manici & Bonora, 2007). The isolates from Serbia also exhibited

a genetic variability, and grouped together with the Italian and reference isolates from Israel and Japan, in three out of four defined sub-clusters, although with different bootstrap support. A confident match has been reported between identification based on hyphal anastomosis pairing and ITS sequence analyses for multinucleate (Gonzales et al., 2001), as well as binucleate *Rhizoctonia* (Gonzales et al., 2001, Sharon et al., 2007; Manici & Bonora, 2007; Fang et al. 2013). However, limited data are available within group AG-A characterization (Manici & Bonora, 2007). Although additional data are needed for an evaluation of the significance of this variability, this is the first report on genetic variability of strawberry *Rhizoctonia* AG-A isolates in Serbia.

In artificial inoculations of strawberry daughter plants, Serbian isolates exhibited uniform pathogenicity with no differences in symptom appearance or intensity. Generally, all isolates caused discoloration of root tissue, but without killing the plants, similarly to Italian strawberry isolates (Manici & Bonora, 2007). Binucleate *Rhizoctonia* AG-A isolates exhibit a wide range of virulence worldwide, and have been described as strong pathogens to non-pathogenic (Botha et al., 2003; Manici & Bonora, 2007; Sharon et al., 2007; Fang et al., 2013), and even reported to occur in mycorrhizal associations (Mazzola, 1997; Sneh et al., 1996). The most common and widely used test for evaluation of the virulence of strawberry binucleate *Rhizoctonia* isolates is the inoculation of strawberry daughter plants, which is a time consuming test requiring a large experimental space. Sharon et al. (2007) reported an additional significant limitation. In their research, the test with inoculation of strawberry daughter plants failed to detect virulence of several isolates from Israel which were causing biomass reduction. In our study, we tested virulence in an additional experiment that aimed to serve as a fast preliminary test by inoculation of detached leaf petioles. This test has already been proposed as suitable to evaluate strawberry cultivar reaction since a correlation between root and petiole reaction has been confirmed (Vico et al., 1995). However, differences in virulence among *Rhizoctonia* AG-A isolates observed in our study could not be statistically confirmed as significant. Nevertheless, this test has successfully confirmed the pathogenicity of all isolates, it is easy to perform, provides reproducible results within merely 4-7 days and, after determining a proper experiment design and number of repetitions, could be used for large scale testing with a large number of isolates.

Strawberry binucleate *Rhizoctonia* AG-A isolates have rarely been experimentally tested for virulence on other plants. In addition to strawberry infection, the Serbian AG-A isolates caused symptoms only on carrot, beans and sunflower, among 14 plants inoculated in the experiment, indicating a narrow host range. Italian strawberry AG-A isolates exhibited similar virulence on strawberry and faba bean seedlings, and low virulence on wheat and brassica (Manici & Bonora, 2007). Binucleate *Rhizoctonia* AG-A has frequently been isolated from hosts other than strawberry, such as bean, peas, tobacco, brassica, potato, wheat and others, but all are reported to be non-pathogenic or with low virulence (Eken & Demirci, 2004; Gurkanli & Ozkoc, 2011; Hua et al., 2014; Unal et al., 2014; Muzhinji et al., 2015; Sharma Poudyal et al., 2015).

The soil-borne nature of *Rhizoctonia* and its worldwide economic significance for strawberry imposes a need for detailed characterization of pathogen populations, in strawberry growing regions in particular. Binucleate *Rhizoctonia* AG-A is the causal agent of black root rot of strawberry in Serbia as shown in this research based on morphological, molecular and pathogenic characteristics of the isolates. In a follow-up study, a larger scale sampling and extensive survey will probably enable detection of additional contributing AG in the *Rhizoctonia* complex causing the strawberry black root rot disease in Serbia and that is a first necessary step in designing efficient control measures.

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Molekularna identifikacija i karakterizacija dvojedarnih *Rhizoctonia* spp. prouzrokovača crne truleži korena jagode u Srbiji

REZIME

Proizvodnja jagode je popularna grana poljoprivrede u Srbiji koja se brzo razvija. Sastav sorti uključenih u proizvodnju podleže stalnim promenama u skladu sa zahtevima tržišta. Jedan od ograničavajućih faktora u proizvodnji jagode širom sveta je crna trulež korena, bolest koju najčešće izazivaju dvojedarne vrste roda *Rhizoctonia*. U Srbiji je poslednjih godina nekoliko puta zabeležena pojava crne truleži jagode uz učestalost koja je iznosila do 30%. Iz biljaka jagode sa simptomima izdvojeni su izolati dvojedarne *Rhizoctonia* AG-A i okarakterisani na osnovu morfološih i molekularnih osobina i patogenosti. Uprkos uniformnim morfološkim osobinama, izolati su ispoljili genetičku varijabilnost na osnovu sekvence ITS rDNK, grupišući se u tri različita filogentska sub-klastera koji su obuhvatali isolate iz Italije, Izraela, Japana i SAD. Izolati dvojedarnih *Rhizoctonia* AG-A poreklom iz Srbije ispoljili su uniformnu virulentnost na jagodi nakon inokulacija živića i fragmenata lisnih peteljki, kao i na sejancima pasulja, mrkve i suncokreta, dok nisu bili patogeni za pšenicu, kukuruz, paradajz, papriku, duvan, krastavac, salatu, grašak, kupus i šećernu repu.

Ključne reči: *Rhizoctonia*; Anastomoza hifa; Dvojedarna AG-A; Analiza sekvenci ITS rDNA; Patogenost; Jagoda