Conventional and real-time PCR assays for detection and identification of *Rhizoctonia solani* AG-2-2, the causal agent of root rot of sugar beet

Mira Vojvodić¹, Dejan Lazić², Petar Mitrović³, Brankica Tanović⁴, Ivana Vico¹ and Aleksandra Bulajić ¹*

¹University of Belgrade, Faculty of Agriculture, Nemanjina 6, Belgrade, Serbia
²East Diagnostics d.o.o, Golovardićeva 32, Belgrade, Serbia
³Institute of Field and Vegetable crops, Maksima Gorkog 30, Novi Sad, Serbia
⁴Institute of Pesticides and Environmental Protection, Banatska 31b, Belgrade, Serbia
*Corresponding author: bulajic_aleksandra@yahoo.com

Received: 22 February 2019
Accepted: 11 March 2019

SUMMARY

Soil-borne fungi belonging to the genus *Rhizoctonia* are considered to be among the most destructive sugar beet pathogens. Although multinucleate *R. solani* AG-2-2 is frequently detected as the main causal agent of root rot of sugar beet worldwide, several binucleate (AG-A, AG-E and AG-K) and multinucleate *Rhizoctonia* (*R. solani* AG-4, AG-5 and AG-8) have also been included in the disease complex. Due to their soil-borne nature and wide host range, the management of Rhizoctonia root rot of sugar beet is highly demanding. Identification of *Rhizoctonia* AG associated with root rot of sugar beet is the essential first step in determining a successful disease management strategy. In this paper we report a highly specific and sensitive real-time PCR protocol for detection of *R. solani* AG-2-2 which showed a high level of specificity after testing against 10 different anastomosis groups and subgroups, including AG-2-1 as the most closely related. Moreover, a similar conventional PCR assay showed the same specificity but proved to be at least a 100 times less sensitive. Future research will include further testing and adaptation of this protocol for direct detection and quantification of *R. solani* AG-2-2 in different substrates, including plant tissue and soil samples.

**Keywords:** *Rhizoctonia solani* AG-2-2; sugar beet; morphology; molecular identification; specific detection, real-time PCR
INTRODUCTION

Sugar beet (Beta vulgaris ssp. vulgaris) is an important crop in European regions with moderate climate where it is grown for its high sugar content (Wibberg et al., 2016). As a crop with high monetary return (Wolf & Verreet, 2002), sugar beet was in 2016 cultivated on over 1.7 million ha in the EU (http://faostat.fao.org/faostat). In Serbia, sugar beet is also considered to be a valuable and profitable crop. In 2017 it was grown on almost 50,000 ha (www.stat.gov.rs). Root diseases of sugar beet, most frequently caused by Rhizoctonia solani, Macrophomina phaseolina, Fusarium oxysporum, Pythium spp., Phytophthora spp., and other agents, are an important constraint to profitable production worldwide, causing yield reductions of up to 50% (Jacobsen, 2006).

In Europe and the USA, the most important sugar beet root diseases are caused by worldwide distributed, soil-borne Rhizoctonia spp., subdivided into numerous anastomosis groups and subgroups (Ogoshi, 1976). Several multinucleate and binucleate Rhizoctonia have been described as sugar beet pathogens. In some sugar beet growing regions, binucleate AG-A, AG-E and AG-K (Strausbaugh et al., 2011; Miles et al., 2013), as well as multinucleate R. solani AG-4, AG-5 and AG-8 have been involved in the Rhizoctonia root rot disease complex (Olaya & Abawi, 1994; Strausbaugh et al., 2011), while multinucleate R. solani AG-2-2 is frequently detected as the main causal agent (Kiewnick et al., 2001; Jacobsen, 2006; Bolton et al., 2010, Abbas et al., 2014). Symptoms related to Rhizoctonia infections of sugar beet mainly include seedling damping-off and root rot with a characteristic presence of circular, necrotic lesions covering large portions of root (Harveson et al., 2009), as well as common above-ground symptoms of wilting and leaf chlorosis. Long-term persistence of R. solani is secured over many years via mycelium and sclerotia in crop debris. Under favorable conditions, sclerotia germinate to infect sugar beet roots (Harveson et al., 2009; Kiewnick et al., 2001; Jacobsen, 2006).

Managing Rhizoctonia root rot of sugar beet is difficult due to the soil-borne nature and wide host range of the pathogen (Allen et al., 1985; Kiewnick et al., 2001). Considering the known differences among AGs regarding host range and fungicide sensitivity, reliable and fast detection and identification of Rhizoctonia AG associated with root rot of sugar beet is the essential first step in developing a successful disease management strategy (Buhre et al., 2009; Amaradasa et al., 2014; Lakshman et al., 2016). Determination of AG composition of Rhizoctonia spp. by morphology-based identification or by isolation, followed by sequencing and phylogenetic analyses, is labor-intensive and time-consuming. Protocols for specific detection based on conventional (end point) PCR are available for several AGs, such as R. solani AG-1, AG-2 (Matsumoto, 2002; Salazar et al., 2000) and AG-3 (Lees et al., 2002). Real-time PCR based on specific detection is also available for several Rhizoctonia AGs, such as R. solani AG-2-1A (Sayler & Yang, 2007), AG-3 (Lees et al., 2002), AG-2-1 (Sturrock et al., 2015), AG-2-2 (Abbas et al., 2014), and AG-8, AG-10, AG-1, and genotypes of R. oryzae (Okubara et al., 2008).

In Serbia, root rot is one of the most important diseases of sugar beet. Until 2000, Fusarium spp. and Macrophomina phaseolina had been considered the most prevalent pathogens of sugar beet in Serbia (Marić et al., 1970). Since then, R. solani has been reported to be widely distributed (Stojšin et al., 2006), causing quantitative and qualitative reductions in yield of sugar beet (Vico et al., 2006). First data on molecular characterization confirmed the presence of R. solani AG 4-HGII (Stojšin et al., 2007) and R. solani AG 2-2 (Stojšin et al., 2011). As population diversity of Rhizoctonia spp. in any particular region may change due to different factors (Fenille et al., 2002; Hua et al., 2014), the main objectives of this study were to: (i) characterize Rhizoctonia isolates infecting sugar beet in Serbia based on morphological features and AG pairing; (ii) identify isolates by sequencing their ITS region of rDNA (ITS1, 5.8S rDNA and ITS2 regions), and (iii) develop a specific and sensitive conventional and real time PCR protocols for detection and identification of R. solani which is pathogenic to sugar beet in Serbia.

MATERIAL AND METHODS

Fungal isolation

Isolates of Rhizoctonia were obtained from symptomatic sugar beet plants collected in the localities of Vašica and Adaševci (Srem District, Serbia) during 2015. Up to 10 randomly distributed samples with symptoms were collected per each locality, then stored at 5°C and processed within 24-48 h. Tissue fragments from the border between necrotic and healthy root tissue were thoroughly washed with tap water for 2 h, surface sterilized for 2-3 min with 50% commercial bleach (2 % sodium hypochlorite), placed on potato dextrose agar (PDA; 200 g potato, 20 g dextrose, 17 g agar and 1 liter of distilled H₂O) and incubated at 24°C
for 3-5 days. *Rhizoctonia*-like colonies were transferred onto fresh PDA and water agar (WA, 17 g agar and 1 liter of distilled H₂O) in order to obtain hyphal tip isolates. The isolates were maintained on PDA slants at 4°C in the Fungal Collection of the Department of Phytopathology, Institute of Phytomedicine, University of Belgrade - Faculty of Agriculture.

### Morphological identification and AG determination

Morphological characterization of *Rhizoctonia* isolates was based on colony appearance and growth rate, as well as hyphal branching pattern and number of nuclei present in young hyphae. Colony appearance was assessed in 7-day old cultures grown on PDA at 24°C in darkness, while growth rate was determined by measuring two perpendicular colony diameters in five cultures per isolate, and calculating an arithmetic average for each isolate. For the growth rate comparison, Student's t-test at 5% and 1% levels were performed (Sokal & Rohlf, 1995). Hyphal branching pattern was observed directly using a compound microscope Olympus CX41. The number of nuclei within hyphal cells was determined using a modified clean slide technique (Kronland & Stranghellini, 1988) after staining with aniline blue in lactophenol or safranin O (Herr, 1979).

Testing for AG grouping was done using the modified clean slide technique (Kronland & Stranghellini, 1988; Martin, 2000) by pairing unknown *Rhizoctonia* isolates with tester isolates of nine different anastomosis groups and subgroups of multinucleate *Rhizoctonia*, AG-1-1C (R62), AG-2-1 (00269), AG-2-2 (01336), AG-3 (R14 1/97 T1), AG-4 HGI (2319), AG-5 (B8), AG-6 (06-01), AG-8 (R28) and AG-9 (CBS970.96) (kindly provided by DrJames Woodhall, University of Idaho, USA, and Dr Véronique Edel-Herman, INRA France). The anastomosis reactions were classified as frequent (more than five fusions observed), weak to intermediate (up to 5 fusions observed) or incompatible (no fusion observed) (Manici & Bonora, 2007).

### Pathogenicity testing

Pathogenicity of two selected Serbian sugar beet *Rhizoctonia* isolates (270-15 and 275-15) was tested using inoculations of non-wounded sugar beet seedlings. Superficially sterilized commercial seeds of sugar beet were placed on PDA slants in 20 cm glass tubes and incubated at 23-25°C in a cycle of 12 h light/12 h darkness. After seed germination and cotyledon development, mycelial plugs (5 mm in diameter) from 7-day old cultures were placed on the roots (mycelial surface face down), while seedlings inoculated with sterile PDA served as negative control. The pathogenicity of the isolates was estimated 7 days post inoculation (dpi). Each isolate was inoculated onto five seedlings, and the experiment was repeated twice. Re-isolations from symptomatic seedlings were performed using the same methods as for fungal isolation.

### DNA amplification and sequencing

For molecular identification of two selected *Rhizoctonia* isolates (270-15 and 275-15), total genomic DNA was extracted from 100 mg of dry mycelium from 5-7-day old cultures grown in potato dextrose broth (PDB) by the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The selected genome region ITS rDNA was amplified using the primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990) (Table 1). Amplification reactions were performed in a total reaction volume of 25 μl, consisting of 12.5 μl 2 × 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 35s 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

| Primer name | Primer sequence (5'-3') | Reference | Amplicon size |
|-------------|-------------------------|-----------|---------------|
| ITS 1F      | CTTGGTGCATTTAGAGGAAGTAA | Gardes & Bruns, 1993 | 700 bp |
| ITS 4       | TCCCTGGCTTTATTGATATGC  | White et al., 1990 | |
| AG-2-2_F    | CAC CTT TTT CTC TTT TTT TAA TCC A | Budge et al., 2009 | |
| AG-2-2_R    | ATA AAT TGG GTT TAT ATT AGA GTT GAG TAG ACA | Budge et al., 2009 | 150 bp |
sequenced in both directions in an automated sequencer (ABI 3730XL Automatic Sequencer Macrogen Inc., Korea), using the same primers as for the 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). 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.

**Conventional and real-time PCR detection**

Protocols for specific detection of Rhizoctonia AG-2-2 based on conventional and real-time PCR were developed and compared. Both protocols are based on a previously published specific primer pair (Table 1) designed for detection of AG-2-2 using TaqMan chemistry (Budge et al., 2009).

Conventional PCR was performed in a total reaction volume of 5 μl, consisting of 2.5 μl 2 x PCR Master mix (Fermentas, Lithuania), 1.3 μl Nuclease-free water, 0.5 μl of both forward and reverse primers (100 pmol/μl, Metabion International, Deutschland) and 0.2 μl template DNA. Amplification conditions were as follows: initial denaturation step of 4 min at 94°C, followed by 35 cycles of 1 min at 95°C, 1 min at 49°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.

Real-time PCR was conducted using a magnetic induction MIC qPCR cycler (Bio Molecular Systems, Australia) in the total reaction volume of 5 μl, containing 0.2 μl of target DNA, 2.5 μl FastGene IC Green 2x qPCR Universal mix (Nippon Genetics Europe Gmbh), 0.5 μl of both forward and reverse primers (100 pmol/μl, Metabion International, Deutschland), and 1.3 μl Nuclease-free water. The amplification program consisted of 95°C for 2 min, followed by up to 40 cycles consisting of 95°C 15 s, 60°C 30 s, and 72°C 2 s. Fluorescence was monitored after each annealing step. Amplicon melting profiles were generated by increasing the temperature of the reaction from 72 to 95°C (0.3°C/s).

Specific detection using both protocols was tested against 10 different anastomosis groups and subgroups of binucleate (AG-A, AG-G, AG-F and AG-U), as well as multinucleate (AG-2-1, AG-3, AG-4-HGI, AG-4-2, AG-6 and Waitea) Rhizoctonia isolates from the Fungal Collection of the Department of Phytopathology, Institute of Phytomedicine, University of Belgrade - Faculty of Agriculture. The sensitivity of both conventional and real-time PCR were compared using 10⁻¹ to 10⁻⁵ dilutions of target DNA. All samples in all experiments were amplified in duplicates or triplicates and all PCRs included NTCs (no template control).

**RESULTS**

**Disease symptoms, pathogenicity and conventional identification**

During 2015, samples were collected from two sugar beet crops with root rot disease incidence estimated at 30%. Only Rhizoctonia-like isolates were recovered from plants exhibiting typical symptoms of Rhizoctonia root rot (Fig 1A, B). A total of 17 hyphal-tip isolates from both localities (8 and 9 isolates from localities of Vašica and...
and Adaševci, respectively) had uniform morphological features, and one isolate from each locality was selected for further studies, i.e. isolates designated as 270-15 (locality of Vašica) and 271-15 (locality of Adaševci) (Table 2).

Pathogenicity testing revealed that both Serbian Rhizoctonia isolates were highly pathogenic, causing prominent root necrosis 7 dpi on all inoculated sugar beet seedlings. Control seedlings showed no symptoms. Isolates were successfully recovered from all symptomatic seedlings thus fulfilling Koch’s postulates.

Both characterized Rhizoctonia isolates from sugar beet had uniform macroscopic and microscopic features (Table 2, Figure 2) with multinucleate nuclear cell condition and typical hyphal branching pattern. The colonies were fast growing, buff to dark brown, with abundant aerial mycelia and visible sclerotia distributed zonally on the periphery (Figure 2A, B). Typical monilioid cells were present on colony surface 3 dpi (Figure 2B), and brown sclerotia 7 dpi (Figure 2C). The isolates differed by their average growth rate (p<0.01), which was 17.08 and 17.38 mm for isolates 270-15 and 271-15, respectively. The presence of hyphal anastomosis was observed in all pairings of both 270-15 and 271-15 isolates against the AG-2-2 tester isolate (isolate 01336). No anastomosis was observed

| Isolate | Year of isolation | Locality    | Colony color | Growth rate | Nuclear condition | Monilioid cells presence |
|---------|-------------------|-------------|--------------|-------------|-------------------|--------------------------|
| 270-15  | 2015              | Vašica      | Brown        | 17.08±0.084 a | >2                | +                        |
| 271-15  | 2015              | Adaševci    | Brown        | 17.38±0.130 b | >2                | +                        |

a, b. Average growth rate (MS±SD) in mm/day - the letter indicates significant difference

Figure 2. *Rhizoctonia solani* AG-2-2: Buff to dark brown colonies with abundant mycelia (A, B), monilioid cells (C), brown sclerotia formation (D).
Mira Vojvodić et al.

after pairings against the AG-1-1C, AG-2-1, AG-3, AG-4 HGII, AG-5, AG-6, AG-8 and AG-9 tester isolates.

**Molecular identification**

Successful amplification of the ITS region of rDNA of both Serbian *Rhizoctonia* isolates resulted in obtaining amplicons of the predicted size of 700 bp, and after sequencing, manually edited consensus sequences of the isolates 270-15 and 271-15 were deposited in the GenBank database (Acc. Nos. MK123309 and MK123311, respectively). Sequence analyses revealed that the isolates share 99.6% similarity (with 3 bp differences), while BLAST analysis revealed the highest nt sequence homology of 99% (100% query coverage) of Serbian sugar beet isolates with over 50 sequences of *R. solani* mainly AG-2-2 but also with some of related AGs from different parts of the world and different host plants. Preliminary phylogenetic analyses (data not shown) confirmed that both Serbian sugar beet isolates belonged to *R. solani* AG-2-2.

**Conventional and real time PCR specific detection**

Using extracted DNA from pure cultures as the template, both conventional and real-time PCR protocols specifically amplified only the isolates of *R. solani* AG-2-2, exhibiting high specificity of both protocols. Using real-time PCR, the isolates 270-15 and 271-15 *Rhizoctonia* AG-2-2 were detected as early as in the 8-10th cycles, which is at least 15 cycles earlier than any other detectable amplification (Figure 3). Melting temperatures of Tm 81.3°C and 81.27°C, respectively, provided clear differentiation from any other test *Rhizoctonia* AG at amplification, thus additionally confirming specific detection. Sensitivity of the conventional vs. real-time PCR differed significantly (Figure 4). Real-time PCR detection was successful in all tested dilutions including the highest of $10^{-5}$, while no products were visible in conventional PCR after amplification of $10^{-3}$ dilution and higher.

![Figure 3](image_url). Primer specificity test for *Rhizoctonia solani* AG-2-2: isolates of *Rhizoctonia* AG-2-1, AG-3, AG-4-1, AG-4-2, AG-6, AG-A, AG-G, AG-F, AG-U, Waitée, Serbian AG-2-2 isolates 270-15 and 271-15 and non-target control, amplified using conventional PCR (A) and real-time PCR (B).
DISCUSSION

The genus *Rhizoctonia* comprises a group of soil-borne and highly heterogeneous, species and isolates, some still with ubiquitous taxonomic status. The AG concept has been adopted for grouping isolates that share some common features, including host range, pathogenicity (Lakshman & Amaradasa, 2014) and fungicide sensitivity (Amaradasa et al., 2014). *Rhizoctonia* AG-2 is one of the most diverse AG (Ogoshi, 1976, 1987; Naito & Kanematsu, 1994; Carling et al., 2002) with several defined subgroups, including AG-2-2, which is a well-established and widespread pathogen in Europe, reported in the Netherlands (Schneider et al., 1997), Germany (Buddemeyer et al., 2004), Spain (Gonzalez et al., 2011), Serbia (Stojšin et al., 2011), Poland (Skonieczek et al., 2016), and Italy (Aiello et al., 2017). The host range of *R. solani* AG-2-2 is wide and besides sugar beet (Kiewnick et al., 2001; Jacobsen, 2006; Bolton et al., 2010, Stojšin et al., 2011, Abbas et al., 2014), it also includes tobacco (Gonzalez et al., 2011), bean (Mora-Umana et al., 2013), potato (Muzhinji et al., 2015), soybean (Ajayi-Oyetunde & Bradley, 2018), onion (Misawa et al., 2017), ornamentals (Aiello et al., 2017), as well as maize (Fähler & Petersen, 2004). Further increase in the significance of *R. solani* AG-2-2 in forthcoming years could therefore be expected, coupled with substantial yield reductions in different crops.

*Rhizoctonia* spp. are considered to be some of the most destructive sugar beet pathogens in Serbia and worldwide and so far the presence of *R. solani* AG-4 and AG-2-2 has been confirmed in Serbia (Stojšin et al., 2011). During 2015, we collected samples from two sugar beet crops with disease incidence estimated at 30% and plants exhibiting damping-off of sugar beet seedlings and roots, crown rot, and foliar blight. Two selected isolates representing a group of hyphal tip isolates from each of the localities were identified and characterized and only the presence of *R. solani* AG-2-2 was detected. In the majority of sugar beet production areas in Europe, *R. solani* AG-2-2 is also the most frequently isolated AG (Buddemeyer et al., 2004; Skonieczek et al., 2016) and it is considered to be favored by narrow crop rotation (Ithurrart et al., 2004).

Serbian isolates obtained in this study exhibited uniform morphological features consistent with published *R. solani* AG-2-2 isolates (Misawa et al., 2015). In pathogenicity testing, our isolates exhibited uniform pathogenicity with no differences in symptom appearance or intensity, and caused seedling necrosis similar to USA and Japan isolates (Carling et al., 2002;...
Bolton et al., 2010). Conventional identification by successful AG pairing with a known tester of AG-2-2 isolate, designated our isolates to be R. solani AG-2-2, which was further confirmed by ITS sequencing and sequence analyses. Two isolates originating from different localities and sugar beet crops exhibited a low level of 0.4% nt difference (3 bp), which correlates with a previously detected level of variability within the AG-2-2 population (Carling et al., 2002).

Fast, specific and sensitive Rhizoctonia detection and AG identification is important and needed in practical, every day disease diagnosis, as well as in decision-making support for implementing control measures. In this paper we report a highly specific and sensitive real-time PCR protocol for the detection of R. solani AG-2-2 in which results can be obtained in a couple of hours. The use of already published primers for specific detection of R. solani AG-2-2 (Budge et al., 2009), and modification of the protocol by using SYBR Green chemistry instead of TaqMan significantly reduced the cost of analyses per sample. All real-time PCR reactions were conducted in a MIC thermal cycler in small reaction volumes of 5 μl recommended by the producer which additionally contributed to cost-effectiveness of the analyses. Abbas et al., (2014) also designed primers for detection of R. solani AG-2-2 in conventional, as well as in real-time PCR, with some limitations in amplicon size or cross-reactivity with closely and distantly related fungi including some Fusarium species. Budge et al. (2009) reported that primers designed in their research, which we also used in our protocols, exhibited a low level of cross-reactivity with R. solani AG-2-1. Nevertheless, in all our experiments, primers showed a high level of specificity after testing against 10 different anastomosis groups and subgroups, including the AG-2-1 isolate from our collection. Moreover, conventional PCR assay with the same primers showed in our study the same specificity but proved to be at least 100x less sensitive, probably due to the previously described limitation of post PCR visualization of amplicons (Lees et al., 2002).

Real-time PCR is a tool which can be successfully used for the fast and specific detection of Rhizoctonia at the species and AG levels, as well as for quantification of a wide range of pathogen DNA concentrations (Lees et al., 2002; Lievens et al., 2006). In this paper we are proposing a sensitive and specific real-time PCR protocol for direct identification and detection of R. solani AG-2-2. Future research will include further testing and adaptation of this protocol for direct detection and quantification of R. solani AG-2-2 in different substrates, including plant tissue and soil samples.

ACKNOWLEDGMENT

This research was funded as part of grants III-43001, III 46008 and TR 31025 received from the Ministry of Education, Science and Technological Development of the Republic of Serbia, and was additionally funded by East Diagnostics d.o.o.

REFERENCES

Abbas, S.J., Ahmad, B., & Karlovsky, P. (2014). Real-time PCR (QPCR) assay for Rhizoctonia solani anastomoses group AG2-2 IIIB. Pakistan Journal of Botany, 46(1), 353-356.

Aiello, D., Guarnaccia, V., Formica, P. T., Hyakumachi, M., & Polizzi, G. (2017). Occurrence and characterization of Rhizoctonia species causing diseases of ornamental plants in Italy. European Journal of Plant Pathology, 148(4), 967-982.

Ajayi-Oyetunde, O.O., & Bradley, C.A. (2018). Rhizoctonia solani: taxonomy, population biology and management of Rhizoctonia seedling disease of soybean. Plant Pathology, 67(1), 3-17.

Allen, M.F., Boosalis, M.G., Kerr, E.D., Muldoon, A.E., & Larsen, H.J. (1985). Population dynamics of sugar beets, Rhizoctonia solani, and Laetisaria arvalis: Responses of a host, plant pathogen, and hyperparasite to perturbation in the field. Applied and Environmental Microbiology, 50(5), 1123-1127.

Amaradasa, B.S., Lakshman, D.K., McCall, D.S., & Horvath, B.J. (2014). In vitro fungicide sensitivity of Rhizoctonia and Waitea isolates collected from turfgrasses. Journal of Environmental Horticulture, 32(3), 126-132.

Bolton, M.D., Panella, L., Campbell, L., & Khan, M.F.R. (2010). Temperature, moisture, and fungicide effects in managing Rhizoctonia root and crown rot of sugar beet. Phytopathology, 100(7), 689-697.

Buddemeyer, J., Pfahler, B., Petersen, J., & Marlander, B. (2004). Genetic variation in susceptibility of maize to Rhizoctonia solani (AC 22IIIB) symptoms and damage under field conditions in Germany. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, 111(6), 521-533.

Budge, G.E., Shurb, M.W., Colyera, A., Pietravallea, S., & Boonhama, N. (2009). Molecular tools to investigate Rhizoctonia solani distribution in soil. Plant Pathology, 58(6), 1071-1080.

Buhre, C., Kluth, C., Bürcky, K., Märlander, B., & Varrelmann, M. (2009). Integrated control of root and crown rot in sugar beet: Combined effects of cultivar, crop rotation, and soil tillage. Plant Disease, 93(2), 155-161.
Carling, D.E., Kuninaga, S., & Brainard, K.A. (2002). Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. *Phytopathology*, 92(1), 43-50.

Fähler, B., & Petersen, P. (2004). Rapid greenhouse screening of maize for resistance to *Rhizoctonia solani* AG2-2IIIB. *Journal of Plant Diseases and Protection*, 111(3), 292-301.

Fenille, R.C., Souza, N.L., & Kuramae, E.E. (2002). Characterization of *Rhizoctonia solani* associated with soybean in Brazil. *European Journal of Plant Pathology*, 108(8), 783-792.

Gardes, M., & Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2(2), 113-118.

Gonzalez, M., Pujol, M., Metraux, J.P., Gonzalez-Garcia, V., Bolon, M.D., & Borrás-Hidalgo, O. (2011). Tobacco leaf spot and root rot caused by *Rhizoctonia solani* Kühn. *Molecular Plant Pathology*, 12(3), 209-216.

Harveson, R.M., Hanson, L.E., & Hein, G.L. (eds.) (2009). *Compendium of beet diseases and pests*. St. Paul, MN: American Phytopathological Society Press.

Herr, L. (1979). Practical nuclear staining procedures for *Rhizoctonia* like fungi. *Phytopathology*, 69(9), 958-961.

Hua, G.K.H., Bertier, L., Soltaninejad, S., & Hofte, M. (2014). Cytospring systems and cultural practices determine the *Rhizoctonia* anastomosis groups associated with *Brassica* spp. in Vietnam. *PLoS ONE*, 9(11), e111750. doi:10.1371/journal.pone.0111750

Ihrurtart, M.E.F., Büttner, G., & Petersen, J. (2004). *Rhizoctonia* root rot in sugar beet (*Beta vulgaris* spp. *altissima*)-epidemiological aspects in relation to maize (*Zea mays*) as a host plant. *Journal of Plant Disease and Protection*, 111(3), 302-312.

Jacobsen, B.J. (2006). Root rot diseases of sugar beet. *Zbornik Matice srpske za prirodne nauke / Matica Srpska Proceedings for Natural Sciences*, 110, 9-19.

Kiewnick, S., Jacobsen, B.J., Braun-Kiewnick, A., Eckhoff, J.L.A., & Bergman, J.W. (2001). Integrated control of *Rhizoctonia* crown and root rot of sugar beet with fungicides and antagonistic bacteria. *Plant Disease*, 85(7), 718-722.

Kronland, W.C., & Stanghellini, M.E. (1988). Clean slide technique for the observation of anastomosis and nuclear condition of *Rhizoctonia solani*. *Phytopathology*, 78(3), 820-822.

Lakshman, D.K., & Amaradasa, B.A. (2014). The pathogen biology, identification and management of *Rhizoctonia* species with emphasis on isolates infecting turfgrasses. *Indian Phytopathology*, 67(4), 327-345.

Lakshman, D.K., Jambhulkar, P.P., Singh, V., Sharma, P., & Mitra, A. (2016). Molecular identification, genetic diversity, population genetics and genomics of *Rhizoctonia solani*. *Perspectives of Plant Pathology in Genomic Era*, 55-89.

Lees, A.K., Cullen, D.Y., Sullivan, L., & Nicolson, M.J. (2002). Development of conventional and quantitative real-time PCR assays for the detection and identification of *Rhizoctonia solani* AG-3 in potato and soil. *Plant Pathology*, 51(3), 293-302.

Lievens, B., Brouwer, M., vanachter, A.C.R.C., Cammue, B.P.A., & Thomma, B.P.H.J. (2006). Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Science*, 171(1), 155-165.

Manici, L.M., & Bonora, P. (2007). Molecular genetic variability of Italian binucleate *Rhizoctonia* spp. isolates from strawberry. *European Journal of Plant Pathology*, 118(1), 31-42.

Marić, A., Rudić, E., & Avdalović, T. (1970). Problem uvenuća *šećerne* šećere repe u nekim rejonima Jugoslavije. *Savremena poljoprivreda*, 18(11-12), 241-252.

Martin, F.N. (2000). *Rhizoctonia* spp. recovered from strawberry roots in central coastal California. *Phytopathology*, 90(4), 345-353.

Matsumoto, M. (2002). Trials of direct detection and identification of *Rhizoctonia solani* AG 1 and AG 2 subgroups using specifically primed PCR analysis. *Mycoscience*, 43(2), 185-189.

Miles, T.D., Woodhall, J.W., Miles, L.A., & Wharton, P.S. (2013). First report of a binucleate *Rhizoctonia* (AG-A) from potato stems infecting potatoes and sugar beet in the Pacific Northwest. *Plant Disease*, 97(12), 1657.

Misawa, T., Kubota, M., Sasaki, J., & Kuninaga, S. (2015). First report of broccoli foot rot caused by *Rhizoctonia solani* AG-2-2 IV and pathogenicity comparison of the pathogen with related pathogens. *Journal of General Plant Pathology*, 81(1), 15-23.

Misawa, T., Kurose, D., & Kuninaga, S. (2017). First report of leaf sheath rot of Welsh onion caused by nine taxa of *Rhizoctonia* spp. and characteristics of the pathogen. *Journal of General Plant Pathology*, 83(3), 121-130.

Mora-Umana, F., Barboza, N., Alvarado, R., Vásquez, M., Godoy-Lutz, G., Steadman, J.R., & Ramirez, P. (2013). Virulence and molecular characterization of Costa Rican isolates of *Rhizoctonia solani* from common bean. *Tropical Plant Pathology*, 38(6), 461-471.

Muzhinji, N., Truter, M., Woodhall, J.W., & van der Waals, J.E. (2015). Anastomosis groups and pathogenicity of *Rhizoctonia solani* and binucleate *Rhizoctonia*
from potatoes in South Africa. *Plant Disease, 99*(12), 1790-1802.

Naito, S., & Kanematsu, S. (1994). Characterization and pathogenicity of a new anastomosis subgroup AG-3 of *Rhizoctonia solani* Kühn isolated from leaves of soybean. *Annual Meeting Phytopathology Society of Japan, 60*, 681-690.

Ogoshi, A. (1976). Studies on the grouping of *Rhizoctonia solani* Kühn with hyphal anastomosis, and on the perfect stages of groups. *Bulletin of the National Institute of Agricultural Sciences, Series C, 30*(3), 1-63.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. *Annual Review Phytopathology, 25*, 125-43.

Okubara, P.A., Schroeder, K.L., & Paulitz, T.C. (2008). Identification and quantification of *Rhizoctonia solani* and *R. oryzae* using real-time polymerase chain reaction. *Phytopathology, 98*(7), 837-847.

Olaya, G., & Abawi, G.S. (1994). Characteristics of *Rhizoctonia solani* and binucleate *Rhizobitis* species causing foliar blight and root rot on table beets in New York State. *Plant Disease, 78*(8), 800-804.

Salazar, O., Julian, M.C., & Rubio, V. (2000). Primers based on specific rDNA-ITS sequences for PCR detection of *Rhizoctonia solani*, *R. solani* AG 2 subgroups and ecological types, and binucleate *Rhizobitis*. *Mycological Research, 104*(3), 281-285.

Sayler, R. J., & Yang, Y. (2007). Detection and quantification of *Rhizoctonia solani* AG-1 IA, the rice sheath blight pathogen, in rice using real-time PCR. *Plant Disease, 91*(12), 1663-1668.

Schneider, J. H. M., Salazar, O., Rubio, V., & Keijer, J. (1997). Identification of *Rhizoctonia solani* associated with field-grown tulips using ITS rDNA polymorphism and pectic zymograms. *European Journal of Plant Pathology, 103*(7), 607-622.

Skonieczek, P., Nowakowski, M., Piszczek, J., Zurek, M., & Maryka, L. (2016). Influence of selected *Rhizoctonia solani* isolates on sugar beet seedlings. *Journal of Plant Protection Research, 56*(2), 116-121.

Sokal, R.R., & Rohlf, F.J. (1995). *Biometry: The principles and practice of statistics in biological research.* 3rd ed. New York, NY: W.H. Freeman and Company.

Stojšin, V.B., Bagi, F.B., Jasnić, S.M., Baláž, F.B., & Budakov, D.B. (2006). *Rhizoctonia* root rot (*Rhizoctonia solani* Kühn) of sugar beet in Province Vojvodina. *Zbornik Matice srpske za prirodne nauke / Matica Srpska Proceedings for Natural Sciences, 110*, 103-108.

Stojšin, V., Budakov, D., Jacobsen, B., Bagi, F., Grimmie, E., & Neher, O. (2011). Analysis of *Rhizoctonia solani* isolates associated with sugar beet crown and root rot from Serbia. *African Journal of Biotechnology, 10*(82), 19049-19055.

Stojšin, V., Budakov, D., Jacobsen, B., Grimmie, E., Bagi, F., & Jasnić, S. (2007). Identification of *Rhizoctonia solani* isolates from sugar beet roots by analysing the ITS region of ribosomal DNA. *Zbornik Matice srpske za prirodne nauke / Matica Srpska Proceedings for Natural Sciences, 113*, 161-171.

Strausbaugh, C.A., Eujayl, I.A., Panella, L.W., & Hanson, L.E. (2011). Virulence, distribution and diversity of *Rhizoctonia solani* from sugar beet in Idaho and Oregon. *Canadian Journal of Plant Pathology, 33*(2), 210-226.

Sturrock, C.J., Woodhall, J., Brown, M., Walker, C., Mooney S.J., & Ray, R.V. (2015). Effects of damping-off caused by *Rhizoctonia solani* anastomosis group 2-1 on roots of wheat and oil seed rape quantified using X-ray Computed Tomography and real-time PCR. *Frontiers of Plant Science, 6*, 461. doi: 10.3389/fpls.215.00461.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725-2729.

Thompson, J.D., Higgins, D.G., & Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*(22), 4673-4680.

Vico, I.M., Janković, D.B., Krstić, B.B., Bulajić, A.R., & Dukić, N.D. (2006). Multinucleate *Rhizobitis* spp. – Pathogen of sugar beet and susceptibility of cultivars under field conditions. *Zbornik Matice srpske za prirodne nauke / Matica Srpska Proceedings for Natural Sciences, 110*, 109-121.

White, T.J., Bruns, T.D., Lee, S.B., & Taylor, J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., & White, T.J. (eds), *PCR protocols: A guide to methods and applications* (pp 315-322). San Diego, CA: Academic Press.

Wibberg, D., Andersson, L., Tzelepis, G., Rupp, O., Blom, J., Jelonek, L. & Dixellius, C. (2016). Genome analysis of the sugar beet pathogen *Rhizobitis solani* AG2-2IIIB revealed high numbers in secreted proteins and cell wall degrading enzymes. *BMC Genomics, 17*, 245. doi: 10.1186/s12864-016-2561-1.

Wolf, P.F.J., & Verreck, J.A. (2002). An integrated pest management system in Germany for the control of fungal leaf diseases in sugar beet: The IPM sugar beet model. *Plant Disease, 86*(4), 336-344.
Konvencionalni i real-time PCR protokoli za detekciju i identifikaciju *Rhizoctonia solani* AG-2-2 prouzrokovala truleži korena šećerne repe u Srbiji

**REZIME**

Smatra se da su gljive iz roda *Rhizoctonia* koje se održavaju u zemljištu, najdestruktivniji patogeni šećerne repe. Mada je širom sveta više jedarna *R. solani* AG-2-2 najčešće označena kao najvažnijii prouzrokovala truleži korena šećerne repe, nekoliko dvojedarnih (AG-A, AG-E i AG-K) kao i više jedarnih *Rhizoctonia* (*R. solani* AG-4, AG-5 i AG-8) takođe mogu da učeštuju u kompleksku bolesti. Kako se održavaju u zemljištu i imaju širok krug domaćina, suzbijanje vrsta *Rhizoctonia* koje izazivaju trulež korena šećerne repe veoma je zahtevno. Identifikacija anastomoznih grupa prouzrokovala oboljenja predstavlja neophodan prvi korak u uspostavljanju uspešnog suzbijanja. U ovom radu razvijen je visokospecifičan i osetljiv real-time PCR protokol za detekciju i identifikaciju *R. solani* AG-2-2 koji se pokazao kao viskospecifičan nakon testiranja koja su obuhvatila 10 različitih AG grupa i podgrupa, uključujući i AG-2-1 kao najsrodniju. Sličan konvencionalni PCR protokol ispoljio je istu specifičnost, ali i najmanje 100 x manju osetljivost. Buduća istraživanja uključuju dalje testiranje i adaptaciju ovog protokola za direktnu detekciju i kvantifikaciju *R. solani* AG-2-2 u različitim uzorcima, uključujući biljno tkivo i zemljište.

**Ključne reči:** *Rhizoctonia solani* AG-2-2; šećerna repa; morfologija; molekularna identifikacija; specifična detekcija; real-time PCR