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Charakterystyka dwujądrowych przedstawicieli rodzaju *Rhizoctonia*

Characteristics of binucleate isolates of *Rhizoctonia* spp.

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wykonana w
Samodzielnej Katedrze Biotechnologii i Biologii Molekularnej
Uniwersytetu Opolskiego

Opole 2016

INTRODUCTION

The genus *Rhizoctonia* spp. is a very diverse group of saprotrophic, pathogenic and mycorrhizal soil fungi. Pathogenic isolates usually cause damages to underground parts of plants, but symptoms of the disease are sometimes visible on the aerial parts, or destruction of whole plant occurs.

The diversity of food preferences of *Rhizoctonia* spp. results in lower quality and yields of many plant species – cereals, ornamentals, vegetables, trees and others. The occurrence of *Rhizoctonia* spp. was reported in over 40 countries, but it is possible that they occur worldwide. The composition of the population is closely linked to climate and species composition in the particular area.

Within the *Rhizoctonia* genus the division based on the number of nuclei in cells occurs. Fungi which are the subject of this study are binucleate *Rhizoctonia* (BNR) with *Ceratobasidium* spp. teleomorphic stage. BNR isolates show pathogenic properties against a great number of plant species. They can cause various kinds of damages, f. ex. damping-off and root rot.

The identification of morphological features of teleomorph stage is very important in the classification of this group of fungi. The BNR teleomorphs are very rare in nature and it is difficult to obtain them in the laboratory conditions. For this reason these fungi are initially characterized on the basis of the vegetative features of anamorphic stage, such as mycelium colour, the number of nuclei in young, actively growing cells and diameters of their hyphae.

BNR have been divided on the basis of the anastomosis reactions into 21 anastomosis groups (from AG-A to AG-U). There are usually isolates with similar food preferences within the group, but the basis for the determining the membership to the group is checking the type of reaction between the hyphae of examined and tester isolates. The molecular analysis of ITS1-5.8S-ITS2 regions of ribosomal DNA and its comparison with sequence database also allows determining the affiliation to AGs.

There is a lot of BNR isolates in Polish soils. They were isolated from sugar beet, cereals and forest nurseries. Despite that BNR, similarly as multinucleate *R. solani,* exhibit pathogenicity against number of plant species, nobody in Poland has not taken the detailed characteristics of these group of fungi. The occurrence of AGs with a wide range of host plants, which includes both trees and crops, may be a problem in agriculture, horticulture and in the wasteland management. In consideration with such a large gap in knowledge it was decided that the aim of this work will be characterization of the representatives of binucleate isolates of *Rhizoctonia* spp. occurring in Poland.

MATERIALS AND METHODS

The study based on the collection of *Rhizoctonia* spp. isolated from woody plants and herbaceous from the area of the southern Poland. Isolates were obtained for the collection of the University of Opole from the collection of the Faculty of Forestry, University of Agriculture in Krakow. The studies also included isolates from the collection of scientific supervisor prof. Ewa Moliszewska and tester isolates for binucleate *Rhizoctonia* spp. from the collection of prof. Mitsuro Hyakumachi from the University of Gifu (Japan).

The cultures of isolates from the collection of *Rhizoctonia* spp. were observed microscopically - the diameters of hyphae and number of nuclei in young cells were measured. Isolates with two- and three nuclei were assigned for further investigation. For comparison purposes, the studies include few isolates of multinucleate *Rhizoctonia* spp. from the University of Opole collection. The growth rates of tested isolates in the dark at 8±1°C, 22±1°C and 30±1°C were measured.

Two methods were used to induce the formation of teleomorphic stage. The first was the transfer of mycelium from the rich in nutrients to the poor substrate. The second method consisted of use a soil additive to the substrate.

For isolation of genomic DNA, the CTAB method was used [Murray and Thompson 1980, Wagner et al. 1987]. PCR reactions were carried out based on the He method [2011]. For the amplification of ITS1-5.8S-ITS2 rDNA regions, ITS1 and ITS4 primers were used [White et al. 1990]. The PCR products were purified and sequenced. Thus obtained sequences were compared with the sequences deposited in GenBank to determine their similarity and belonging to the anastomosis groups. The obtained results were checked by pairing tester isolates with a known affiliation to anastomosis groups by Kronland and Stanghellini [1988] and Moliszewska [2002] methods.

The phylogenetic tree, which illustrate phylogenetic relationships between tested isolates, was constructed based on the sequence regions ITS1-5.8S-ITS2 rDNA by the neighbor joining method using MEGA6 program.

Differences in the ability to produce pectinolytic enzymes allows the differentiation of fungi within the group. To induce the production of pectinolytic enzymes, selected isolates were cultured in a FNA medium and then in the pectin medium. The obtained filtrates were subjected to native electrophoresis in polyacrylamide gel [Sweetingham et al. 1986].

To determine the ability to thiamine synthesis, tested BNR isolates were cultured on liquid Czapek-Dox and Czapek-Dox with the addition of vitamins in concentration 10^{-5} M.

The obtained mycelium was filtrated, dried, and the dry weight was measured. Auxotrophy was determined using the Carling et al. [1987] formula.

BNR are organisms with a variety of food preferences. The different plant species were selected to determine the effect of BNR on their growth and health. The plants selected for the study were: sugar beet, cucumber, wheat, carrot and sage. Effect of *Rhizoctonia* spp. isolates on the growth and development of selected model plant species was determined in the laboratory conditions by Moliszewska [2000, 2009] method. On the basis of the results the index of infected plants was calculated [Burgieł 1980]. Plant lengths of healthy seedlings were measured to determine the possible influence of *Rhizoctonia* isolates on plant growth.

Some pathogenic *Rhizoctonia* isolates are able to secrete toxic metabolites. To obtain supernatants with these metabolites, isolates grown on liquid Czapek-Dox medium. Sugar beet seedlings were placed in petri dishes containing post-culture filtrates. The degree of seedling destruction was evaluated.

Pathogenic fungi are able to degrade plant tissues and cells by pectinolytic enzymes. To stimulate the production of these enzymes, BNR isolates were grown on medium containing citrus pectin. After the incubation time the mycelia were separated and fragments of healthy sugar beet hypocotyls were placed in the filtrates. After 24 and 48 hours, the parts of the plants were stained with toluidine blue. Tissues were observed under light microscope to determine the intensity of the colour demonstrating the ability of BNR to degrade pectin.

BNR isolates were tested for sensitivity on fungicides containing active substances such as thiram, carbendazim and thiophanate-methyl. Mycelia were placed on plates containing PDA medium with tested fungicides at concentrations of 10, 50 and 100 ppm. The colony diameters were measured every 24 hours. To calculate the growth inhibition by the tested chemicals, measurements were calculated according to the Abbott formula [Kowalik i Krechniak 1961].

In another experiment the effect of humic acids on the development of BNR were examined. Humic acids were obtained by Schnitzer [1978] method with the modification proposed by Man et al. [2013]. The mycelium disks were placed on the PDA medium enriched with the addition of the examined acids [Moliszewska and Pisarek 1996]. The diameters of the colonies were measured at the 24-hour intervals. The results were calculated according to the Abbott formula [Kowalik i Krechniak 1961].

Rhizoctonia spp. are closely related to the soil environment, thus their ability to grow and spread in different types of substrates were verified. Mycelia were placed in containers with tested substrates. When isolates overgrown the entire surface of the control substrate,

sterile toothpicks were inserted into the tested substrates. After 48 hours toothpicks were removed and plated onto Petri dishes with selection medium. The presence of mycelium were checked under a light microscope [Paulitz i Schroeder 2005].

RESULTS

The group of tested BNR isolates, which was the subject of presented studies, showed a wide morphological diversity – mycelium color, type of growth and ability to growth on a solid growth medium.

Differences also were manifested in a growth rate – the majority of isolates preferred the temperature about 22°C, only single isolates grew faster in 30°C. It was also observed that BNR were able to grow in low temperatures (about 8°C) although it was significantly slower.

Some isolates from tested collection were not capable to produce thiamine, however, there were no relationship between this feature and their memberships to anastomosis groups.

Comparison of ITS1-5,8S-ITS2 sequences of rDNA of tested isolates with sequences deposited in GenBank indicated their affiliation to three AGs – AG-A, AG-E and AG-K. Their membership to AGs was confirmed by anastomosis reactions with tester isolates.

Not all isolates had an equal number of nuclei in cells – a group of fungi produced cells with diverse number of nuclei which ranged from 2 to 4, where average number of nuclei was close to 3. These specific isolates proved to belong to AG-E. It can be assumed that all isolates with three nuclei in cells belong to this group, which can greatly facilitate preliminary identification of binucleate *Rhizoctonia* spp. isolates. This feature can also be a prerequisite for separation of two subgroups within the AG-E: a subgroup of bi-nucleate strains and a subgroup of three-nucleate strains.

Isolates from AG-A and AG-K were close related to each other (the ITS1-5,8S-ITS2 sequences identity varied from 89-95%), while AG-E isolates showed a much lower level of identity of these sequences (78-88%) and were more closely related to f. ex. AG-F and *R. solani.*

Tested BNR isolates varied in the pathogenicity – some of them didn't show pathogenic properties to model plants – sugar beet, cucumber, wheat, carrot and salvia. Others were very pathogenic, although the pathogenicity varied depending on the plant species. Sugar beet was a plant species especially sensitive to BNR infection. The diversity of virulence occurred between anastomosis groups, but also it was observed between isolates from the same group. It was also demonstrated that some isolates may affect on plant growth by stimulating or slowing their development. The tested BNR can secrete extracellular metabolites (f. ex. pectic

enzymes) which are able to degrade the plant tissues components, causing damage and allowing to infect a previously undamaged plants. Tested BNR isolates produced less of these substances than representatives of *R. solani* which are generally considered to be stronger pathogens than BNR. The profile of pectinolytic enzymes, obtained in this study, do not allow differentiating BNR within the anastomosis groups.

The susceptibility of BNR on the humic substances and fungicides were diverse. They were able to spread in different types of soil.

Non-pathogenic BNR usually developed slower than *R. solani* and in two-organism cultures were dominated by pathogenic forms both *R. solani* and BNR.

CONCLUSIONS

On the basis of experiments and observations the following conclusions can be drawn:

1. BNR strains isolated from the plants in Poland belongs to three anastomosis groups – AG-A, AG-E and AG-K. AG-A is identified for the first time in Polish mycobiota.
2. The presence of an average of three nuclei in the cells of *Rhizoctonia* spp. hyphae indicates their likely belonging to the AG-E. This feature should be considered as a new diagnostic clue, keeping in mind that in this group there are also binucleate forms.
3. It is possible to divide AG-E into two groups differing in the number of nuclei in actively growing cells (average of 3 or an average of 2 nuclei). This suggestion, however, requires further study.
4. Strains from groups AG- A and AG-K are closely related, they have similar sequences in the region ITS1-5,8S-ITS2 rDNA profiles and pectinolytic enzymes, but they don't anastomose to one another while the strains from AG-E are closer related to *R. solani* than AG-A and AG-K.
5. Tested BNR isolates show a high pathogenicity to sugar beet seedlings, and diverse to other tester plants. They can also adversely affect the growth of plants without causing disease symptoms, while their degree of pathogenicity to the plant model is very diverse and is not related to participation to anastomosis group,
6. Few binucleate *Rhizoctonia* spp. may have a positive effect on plant growth, which is particularly manifested in the formation of longer root system, there were no connection with belonging to the anastomosis group.
7. In the tested collection, only one isolate showed no virulence against all model plants.

8. The majority of BNR are sensitive to common fungicides such as thiophanate-methyl and less thiram, but there are also the strains resistant to fungicides. Such studies should be conducted in a wider range, as they are essential for the selection of appropriate measures in the protection of plants against pathogenic BNR.
9. The tested binucleate *Rhizoctonia* spp. (BNR) are sensitive to presence of humic substances in the substrate, mostly in the form of fulvic acids, which may affect their ability to proliferation and survival in the soil environment.

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