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Małgorzata NABRDALIK[1*] and Katarzyna GRATA[1]

THE ROLE OF *Pseudomonas fluorescens* IN THE PROCESS OF *Rhizoctonia solani* GROWTH INHIBITION

UDZIAŁ *Pseudomonas fluorescens* W ZAHAMOWANIU WZROSTU *Rhizoctonia solani*

Abstract: The aim of conducted research was to determine influence of metabolites produced by *Pseudomonas fluorescens* on the growth of 4 phytopathogenic strains of *Rhizoctonia solani* marked R1, R2, R3 and R4 which infect sugar beetroot. The antagonistic properties were assessed with the culture-plate method on PDA medium for *P. fluorescens* cultured for 4, 6 8, 10 and 24 hours at 25 ^{o}C for 5 days. The fungistatic activity of *P. fluorescens* was determined against the growth rate index and the rate of mycelial growth inhibition. Obtained results prove, that *Rhizoctonia* spp. strains were both sensitive and resistant to metabolites produced by *P. fluorescens*. The highest inhibition of the linear growth of mycelium has been observed for *R. solani* R1 and R4 strains. In both cases the highest inhibition, reaching almost 60 %, has been recorded for the trials cultured for 4 hours and the lowest, amounting ca. 30 % after 24 hours of culturing. However, the other strains of *R. solani* marked R2 and R3 were resistant to the metabolites produced by *P. fluorescens* regardless of the length of culturing.

Keywords: antifungal activity, *Pseudomonas fluorescens*, *Rhizoctonia solani*, growth rate index

Introduction

Rhizoctonia solani Kühn is one of the most commonly recognised soil pathogens. It can persist in soil for a long time due to durable sclerotia and saprophytic manner of mycelial growth which infects host plants. *Rhizoctonia solani* is a destructive plant pathogen and can cause damage worldwide on more than 142 plant species, including many agricultural and horticultural crops. *Rhizoctonia solani* is an heterogeneous species composed of subspecific groups called anastomosis groups (AGs) defined on the basis of hyphal anastomosis reactions [1–2]. On sugar beet, *R. solani* is responsible for diverse pathologies. Isolates from AG-2 and AG-4 are known to be pathogenic on roots of adult plants and on seedlings, respectively. Additional anastomosis groups

[1] Independent Chair of Biotechnology and Molecular Biology, Opole University, ul. kard. B. Kominka 6a, 45–032 Opole, Poland, phone: +48 77 401 60 56.

* Corresponding author: mnabrdalik@uni.opole.pl

R. solani can be virulent on seedlings or have been isolated from soils or sugar beets in diseased fields [3–4]. In Poland, there have been the following anastomosis groups of *R. solani* Kühn recognized at sugar beetroot plantations: AG 5, AG 1 IB, AG 2-1, AG 2-2 IIIB, AG 4 HG II and AG 11, which infect mostly beetroot seedlings [5]. Economic losses were estimated to average 2 % in the United States; however, damage can vary greatly (0 to 50 %) from field to field depending on cropping history and environment [6-7]. There are limited possibilities to protect damaged plant roots with chemical substances, therefore the only way to reduce the danger of infecting the host plant by *R. solani* is an application of an appropriate agrar procedure, a selection of tolerant types of plants as well as searching for alternative methods of protection, such as biological control employing microorganisms [8]. Biological control method employ microorganisms being the natural antagonists of plants pathogens [9]. Such microbes are very often isolated from the same natural environment where they will be applied again.

It is worth considering bacteria residing in a rhizosphere of cultivated plants when searching for the appropriate bacteria strains. As shown in research papers, *P. fluorescens* may be useful as a potential antagonist towards phytopathogenic fungi. Certain members of the *P. fluorescens* have been shown to be potential agents for the biocontrol which suppress plant diseases by protecting the seeds and roots from fungal infection. They are known to enhance plant growth promotion and reduce severity of many fungal diseases [10–12]. It is believed that biological activity of *P. fluorescens* results from their ability to produce biologically active compounds such as: hydrogen cyanide, salicylic acid, siderophores, lytic enzymes, proteases and secondary metabolites revealing strong antifungal properties – pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol [13–15]. Hass and Défago [12] reviewed the mechanisms by which *P. fluorescens* control pathogenic microorganisms in detail. During root colonization, these bacteria produce antifungal antibiotics, elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors. Before engaging in these activities, biocontrol bacteria go through several regulatory processes at the transcriptional and post-transcriptional levels. In this way, competitive exclusion of pathogens as the result of rapid colonization of the rhizosphere by *P. fluorescens* may also be an important factor in disease control.

In the research, the activity of soil strain *P. fluorescens* against four selected *R. solani* strains isolated from infected sweet beetroot roots has been examined.

Materials and methods

In the experiment, a fungistatic activity of *P. fluorescens* against 4 strains of *R. solani* marked R1, R2, R3 and R4 has been assessed. The strain *P. fluorescens* was isolated from soil and identified with the use of ID32GN tests (bioMérieux) and Bergey's Manual of Systematic Bacteriology [16]. The strains of tested fungi were isolated from the infested bulbs of sugar beetroot and diagnosed on the basis of their macro- and microscopic features.

The bacteria were cultured in the broth medium for 48 hours at 30 ^{o}C. Next, the broth was inoculated with the suspension of 10^6 cfu/ml density and incubated for the time

period from 4 to 24 hours. After the incubation it was centrifuged at 10 000 rpm and obtained supernatant underwent further analysis.

Conducted tests employed a culture-plate method applied on PDA growth medium consisting of (g/dm^3): glucose 20.0, potato extract 4.0, agar 15.0. Tested growth media were inoculated with supernatants obtained after 4, 6, 8, 10 and 24 hours of culturing of *P. fluorescens* rods. Next, the media were inoculated with 10 mm discs overgrown with 7-days old mycelium of tested *R. solani* strains. The control trials contained only tested *R. solani* strains with no addition of supernatant. All plates were incubated at 25 ^{o}C for 5 days. The diameters on the plates were measured every day until the mycelium of *R. solani,* in the control trial, reached the edge of the plate. The experiment was conducted in six replicates, where one trial was represented by one culturing plate with the growth medium and the mycelial disc.

The influence of metabolites produced by *P. fluorescens* on the growth of *R. solani* strains was determined against the growth rate index, calculated according to the formula [17]:

$$T = \frac{A}{D} + \frac{b_1}{d_1} + \frac{b_2}{d_2} + \ldots + \frac{b_x}{d_x}$$

where: T – the growth rate index,
A – is a mean value of diameter measurements [mm],
D – is the length of the experiment (number of days),
b_1, b_2, b_x – denote an increase in a diameter size since the last measurement,
d_1, d_2, d_x – are the number of days since the last measurement.

The fungistatic properties of the supernatant have been assessed on the basis of the linear growth inhibition of the fungus.

Statistical significance was determined using an analysis of variance (ANOVA) followed by Duncan's test. Values were considered significantly different at $p < 0.05$.

Results and discussion

The paper presents a pilot research which tests *P. fluorescens* strain against its ability to synthesize exocellular metabolites possessing fungistatic abilities in relation to phytopathogenic strains of *Rhizoctonia* spp. Conducted tests revealed the direct influence of metabolites produced by *P. fluorescens* on the growth rate of the fungi under study. It has been noted, that an addition of the supernatant collected at different development phases of bacteria prompted responses which differed for every tested strain. Obtained results are presented by the values of the growth rate index of the mycelium (including the mycelial growth in time) and the degree of the linear growth inhibition of fungi. On the basis of obtained results and statistical analysis, strains of *Rhizoctonia* spp. under study may be divided into two groups due to a lack of statistically significant differences between the strains in test trials. Strains *R. solani* R1 and R4 may be described as sensitive strains, whereas strains *R. solani* R2 and R3 as resistant (Table 1).

Table 1

Influence of *P. fluorescens* on the growth rate index of tested fungi

Culturing time [h]	*R. solani* R1		*R. solani* R2		*R. solani* R3		*R. solani* R4	
	control	trial	control	trial	control	trial	control	trial
4	99.53^{a}	36.67^{a}	94.10^{a}	94.22^{a}	95.35^{a}	67.33^{a}	99.28^{a}	36.86^{a}
6	99.28^{a}	43.14^{ab}	93.92^{a}	94.47^{a}	95.44^{a}	95.20^{b}	98.89^{a}	45.97^{b}
8	98.42^{a}	50.92^{b}	93.94^{a}	95.60^{b}	93.78^{a}	94.24^{b}	98.78^{a}	45.58^{b}
10	97.69^{a}	49.25^{ab}	93.41^{a}	93.70^{a}	93.85^{a}	93.09$_{b}$	98.19^{a}	51.39^{b}
24	99.97^{a}	54.22^{b}	95.42^{a}	95.95^{b}	98.17^{a}	99.39^{b}	100.64^{a}	70.44^{b}

Different letters indicate significant differences (ANOVA, $p < 0.05$, Duncan's test).

Presented paper shows that prolonging the culturing time and at the same time increasing the amount of metabolites do not affect the inhibition rate of the linear growth index. For the strains of *R. solani* R1 and R4 described as sensitive strains the reduction rate of the growth rate index decreases as the culturing time of bacteria is longer (Table 1). The measured values of the indexes for the control trials employing these strains ranged between 97.69 and 100.64, while for the proper trials they amounted from 36.86 to 70.44. The highest inhibition of the growth rate index of *R. solani* R1 was noted in case of 4 and 6-hour culturing of *P. fluorescens* and amounted 63.16 % and 56.55 % respectively. The highest reduction in the growth rate index of *R. solani* R4 was recorded after inoculation with the bacteria cultured for 4, 6 and 8 hours. The amounts obtained are: 62.87 %, 53.51 % and 53.85 % respectively. Prolonging the culturing time did not contribute to a higher reduction of the growth rate index. Therefore, for the both strains *R. solani* R1 and R4 the reduction of the growth rate index was the lowest in case of metabolites obtained after 24-hours of culturing of *P. fluorescens* (45.76 % and 30.01 % respectively) (Fig. 1). For the strains presented in

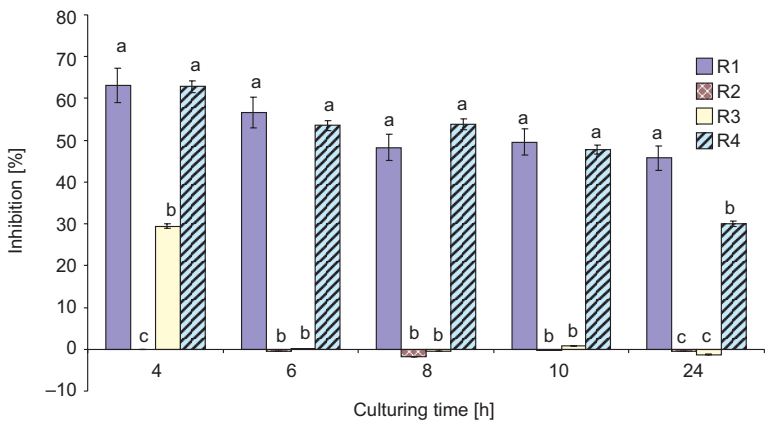

Fig. 1. Reduction of the growth rate index of tested fungi. Different letters indicate significant differences (ANOVA, $p < 0.05$, Duncan's test)

the paper and described as resistant, *ie R. solani* R2 and R3 (Table 1), the values of the growth rate indexes in the control trials ranged between 93.41 and 98.17, and for the proper trials they amounted from 67.33 to 99.39. Recorded values of the growth rate indexes were so high that in most trials their reduction was not noted. Only in case when the culturing medium was inoculated with the bacteria grown for 4 hours, the reduction of the mycelial growth index, lower than 30 %, was noted for *R. solani* R3 (Fig. 1).

The negative values of the reduction of the growth rate index noted during conducted research prove, that metabolites produced by *P. fluorescens* stimulate the mycelial growth of *R. solani* R2 and R3. It would be impossible to draw such conclusion by only analysing the linear growth inhibition of the mycelium (Fig. 2). The chart presents clearly, that introduction of the metabolites produced by *P. fluorescens* did not inhibit the mycelial growth of *R. solani* R2 at all, while the growth of strain R3 was inhibited in 25 % after introduction of bacteria cultured for 4 hours. Significantly higher values were noted for *R. solani* R1 and R4 strains. For the both strains, it has been observed that the longer the bacteria were cultured for, the lower the inhibition rate of the linear growth of mycelium was. The values of noted reduction amounted between 60 % when applying bacteria cultured for 4 hours to 42 % (for *R. solani* R1 train) and 27 % (for *R. solani* R4 strain) when introducing the bacteria cultured for 24 hours.

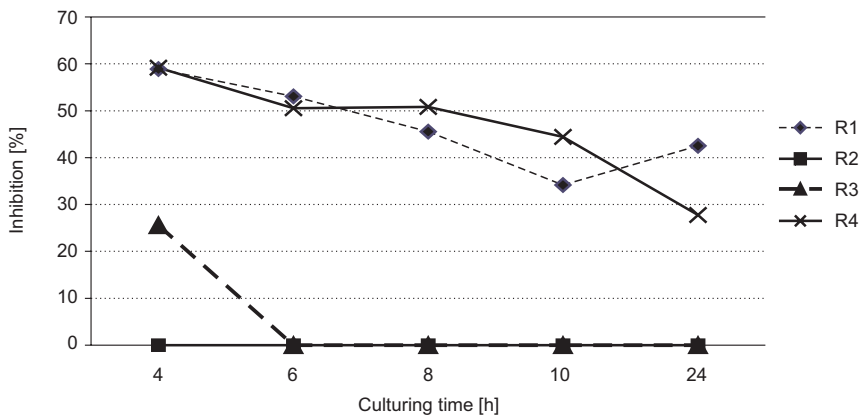

Fig. 2. Influence of *P. fluorescens* on the linear growth of *R. solani* strains

Conducted pilot research showed varied and selective activity of *P. fluorescens* strain against indicative fungi development and thus it is difficult to identify clearly its phytopathogenic activity against soil fungi. Also some differences in the growth rate inhibition of phytopathogenic fungi caused by strains of *P. fluorescens* have been obtained by Jankiewicz [15]. The value of inhibition rate for *P. fluorescens* F1 amounted between 30–80 % and for the strain *P. fluorescens* F2 between 40–90 %. Singh and Sinha [18] found that *P. fluorescens* of higher rate (8 g/dm^3) was highly effective in reducing disease severity (60.0 %) and incidence (35.6 %) and increasing grain yield (33.8 %) and 1000-grain weight (12.9 %). In this study, the lower rate of the

bio-agents though effective against the disease but was found inferior as compared to higher rates. Grosch et al [19] analyzed two strains of *P. fluorescens*, B1 and B2, were evaluated in a growth chamber and in the field against *R. solani* in potato and in lettuce. The greatest disease suppression effect on potato was achieved by strain B1 (37 %), followed by B2 (33 %), whereas the marketable tuber yield increased up to 12 % (B1) and 6 % (B2). Whereas, research by Stachowiak and Dach [20] show that strains of *R. solani* are resistant to bacteria isolated from compost. It seems that inhibitory activity depends on both bacterial strain and indicative fungus. It may result from different sensitivity to antifungal substances produced by bacteria and concentration of such metabolites in the culturing medium. Selective activity of *P. fluorescens* and other bacteria against the development of phytopathogens has been described in many research papers [10, 21–28]. It has been proved that antifungal activity of *P. fluorescens* is closely related to antibiotics and lytic enzymes production. Hammer et al [14] proved, that *P. fluorescens* BL915 strain which produces pyrrolnitrin is an efficient micro-organism in the process of biological control of *R. solani* development, whereas Corbell and Loper [13] showed in their research that the growth inhibition of *R. solani* depends on pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol and hydrogen cyanide. They employed *P. fluorescens* Pf-5 and proved that mutants of Pf-5 ApdA$^-$ produced significantly lower amounts of the above substances and did not inhibit the growth of *R. solani.* Among fourteen strains of *P. fluorescens* tested by Nagarajkumar et al. [29], one marked PfMDU2 was most efficient in terms of mycelial growth inhibition of *R. solani.* At the same time, tested strain showed high activity of β-1,3-glucanase and production of salicylic acid, siderophore and hydrogen cyanide. The authors proved in their research that antifungal properties of *P. fluorescens* depend on the level of substances mentioned above. Vivekananthan et al. [30] proved, that the enhanced expression of defence-mediating lytic enzymes chitinase and β-1,3-glucanase of *P. fluorescens* FP7 may collectively contribute to suppress the anthracnose pathogen, leading to improved yield attributes. Nielson and Sörensen [31] demonstrated that isolates of *P. fluorescens* antagonistic to *R. solani* and *Pythium ultimum,* produced endochitinase and chitobiosidase. Detailed studies of three selected isolates showed that extracellular release of endochitinase activity also took place in stationary phase (corresponding to 25–50 h of incubation). These results differ from author's own results as the highest antifungal activity was noted after the application of supernatants obtained after 4 and 6 hours of culturing, which corresponds to the early logarithmic growth phase. It allows the authors to assume that since in own research there has been no inhibitory activity of *P. fluorescens* R2 and R3 strains recorded, then the strains are not active in terms of β-1,3-glucanase, salicylic acid, hydrogen cyanide and siderophore as well as do not produce pyrrolnitrin.

Conclusions

To sum up, it has been stated that *P. fluorescens* shows antagonistic activity to different strains of *R. solani.* Their potential in suppressing fungal growth should be exploited as a complement or an alternative to chemical control for sheath blight disease

in plants. Significant differences in obtained results indicate that prior to field trials, extended laboratory tests including various strains of *P. fluorescens* are required. It should be noted that, there are some restrictions which may hinder a widespread application of this bacteria in the process of biological control in agriculture. The most important factor concerning their application is variable efficiency of *P. fluorescens* under field conditions, in which the inhibition of phytopathogens growth results from cooperation and complementarity of different mechanisms.

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UDZIAŁ *Pseudomonas fluorescens* W ZAHAMOWANIU WZROSTU *Rhizoctonia solani*

Samodzielna Katedra Biotechnologii i Biologii Molekularnej
Uniwersytet Opolski

Abstrakt: Celem podjętych badań było określenie wpływu metabolitów *Pseudomonas fluorescens* na wzrost 4 fitopatogennych szczepów buraka cukrowego *Rhizoctonia solani* oznaczonych jako R1, R2, R3 oraz R4. Ocenę właściwości antagonistycznych metabolitów przeprowadzono metodą hodowlano-płytkową na podłożu PDA dla 4, 6, 8, 10 i 24 godzinnych hodowli *P. fluorescens*. Hodowle prowadzono w temperaturze 25 ^{o}C przez 4–7 dni. Na podstawie indeksu tempa wzrostu oraz stopnia zahamowania wzrostu grzybni określono aktywność fungistatyczną *P. fluorescens*. Wyniki doświadczenia wskazują, że wśród badanych szczepów *Rhizoctonia* spp. były zarówno szczepy wrażliwe, jak i oporne na działanie metabolitów *P. fluorescens*. Największą inhibicję rozrostu liniowego grzybni zaobserwowano dla szczepów *R. solani* R1 oraz R4. W obu przypadkach najwyższe, prawie 60 % zahamowania wzrostu grzybni uzyskano dla 4-godzinnej hodowli, a najniższe w granicach 30 % dla hodowli 24-godzinnej. Natomiast szczepy *R. solani* R2 i R3 były oporne na działanie metabolitów *P. fluorescens* niezależnie od wieku hodowli.

Słowa kluczowe: aktywność przeciwgrzybowa, *Pseudomonas fluorescens, Rhizoctonia solani,* indeks tempa wzrostu