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THE ROLE OF EXTRACELLULAR METABOLITES PRODUCED BY BACTERIA IN THE PROCESS OF FUNGI GROWTH INHIBITION

UDZIAŁ ZEWNĄTRZKOMÓRKOWYCH METABOLITÓW BAKTERYJNYCH W ZAHAMOWANIU WZROSTU GRZYBÓW

Abstract: In the process of biological control of plants pathogens, special biological preparations containing cells of microorganisms or their metabolites are applied. The aim of conducted research was to assess the effect of *Bacillus subtilis* culture or its supernatant on the mycelial growth of *Fusarium culmorum*. The antagonistic properties of *B. subtilis* were assessed with the culture-plate method. The research was conducted on two different media, containing glucose or sucrose, in order to consider the effect of the source of carbon on the inhibitory process. The culturing process was conducted at 25±2ºC for 7 days and every 2-3 days the diameter of the mycelium was measured while the fungistatic activity of *B. subtilis* was determined against the mycelial growth rate index. The obtained results showed that the measured values of the growth rate index in the control trials were similar regardless of the carbon source in the medium. In tested trials no significant inhibition of the mycelial growth have been noted in the presence of glucose. Measured values of the growth rate index in tested trials were lower of 0.2-1.7 units compared with the control trials. The growth inhibition in the cultures on the medium with sucrose obtained 20% with an addition of the supernatant and 40% with an addition of *B. subtilis* culture.

Keywords: *Bacillus subtilis*, *Fusarium culmorum*, antifungal activity

Introduction

In recent years, there has been a growing interest in a potential application of the nonpathogenic microorganisms isolated from natural habitats in the process of plant protection. The microorganisms may be an alternative to chemical substances which according to the EU Directives [1] should be systematically eliminated from use in order to reduce their level in the environment. One of the reasons for such policy is the fact that pesticides are not selective when applied and what is more they may reduce biological variety in the agricultural production systems and their particles remaining in the field crops may pose hazard to consumers' health. Another issue is the fact that pathogens have the ability to develop resistance against pesticides. Therefore, the research has been conducted in order to find microorganisms which could protect plants against diseases. Such microorganisms belong to the group of plant growth promoting microorganisms (PGPR) which synthesize enzymes hydrolyzing cell wall of fungi. In consequence, the process leads to protoplasts fusion and degradation of phytopathogens. The microorganisms in rhizosphere may induce plant's resistance against diseases. The factors activating such reaction are chemical compounds called elicitors, which are recognized by specific plants receptors and induce biochemical defense reactions. A highly important aspect of such reaction is the fact that it is potentially non-specific in relation to the pathogen, contrary to classic (direct) methods of biological control, which apply the substance usually active

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against one or at most several pathogens. Among microorganisms showing such properties there is a group of bacteria *Bacillus*, which produce metabolites very actively. Many species of *Bacillus* spp. are widely known for their antagonistic properties against the following fungi: *Alternaria, Fusarium, Rhizoctonia, Phytophthora* and *Pythium*, but also as factors stimulating plants' growth [2-5]. The application of biological methods may reduce the amount of applied synthetic pesticides and in some cases may lead to their elimination as chemical substances are dangerous to biological balance in the soil as well as the quality of plants grown.

The aim of this paper was an assessment of fungistatic properties of *B. subtilis* against strain *F. culmorum,* one of the most frequently isolated plant pathogen, causing many diseases called fusariosis.

Materials and methods

In the research, a fungistatic activity of *B. subtilis* for the growth control of *F. culmorum* has been assessed. The bacteria were grown in the nutrient broth at 30ºC for 48 hours. The suspension of bacterial cells at an optical density of 1.0 or 2.0 was used to inoculate the tubes with nutrient broth and incubated for 24 hours. For the further studies the supernatants obtained from the bacterial cultures were used as well as bacterial cultures.

The antagonistic activity of *B. subtilis* against *F. culmorum* was assessed *in vitro* tests with a dual culturing-plate method using two different sources of carbon - glucose or sucrose. In the experiment the following culturing media were used: PDA consisting of [g/dm^3]: glucose 20.0, potato extract 4.0, agar 15.0 and Czapek consisting of [g/dm^3]: sucrose 30.0, $MgSO_4 \times 7H_2O$ 0.5, KH_2PO_4 1.0, KCl 0.5, $NaNO_3$ 3.0, $Fe_2(SO_4)_3 \times 7H_2O$ 0.01, agar 15.0. In the test trials the media were inoculated with either 0.5 cm^3 of the bacterial culture or 0.5 dm^3 of the supernatants. Next, the media were inoculated with 10 mm discs overgrown with 7-days old mycelium of tested *F. culmorum* strains. The control trials contained only tested strains of *F. culmorum*. All plates were incubated at 25ºC for 7 days. The diameters of the colony on the plates were measured every 2-3 days for the period of 7 days until the mycelium of *F. culmorum* in the control trial, reached the edge of the plate. The experiment was run in triplicate, where one replicate was represented by one culturing plate with the culturing medium and one mycelial disc.

The influence of metabolites produced by *B. subtilis* on the growth of *F. culmorum* was determined against the growth rate index, calculated according to the formula below [6]:

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

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

Results and discussion

B. subtilis is acting as an antagonist by competing for nutrients and space as well as by producing hydrolases (amylase, protease, pullulanase, chitinase, xylanase, lipase) and natural substances such as: subtilisin, fengycin, bacilysocin, surfactin or iturine [7-10]. Therefore, in the pilot research it has been assessed how the bacterial culture or the cell free supernatant containing metabolites produced by *B. subtilis* affects the growth of *F. culmorum.* Additionally, the density of bacterial inoculum and the type of the medium (source of carbon) were taken into account.

In the conducted tests no significant differences were noted in obtained values of the growth rate index in the control trials. Regardless of the source of carbon in the culturing medium the growth rate index amounted over 43 units (Figs. 1, 2). However, the control trials have proved that the activity of *B. subtilis* was different depending on the parameters analysed in the experiment. Therefore, the value of the growth rate index for the supernatant was similar to the value noted in the control trial when the optical density of the inoculum was 1.0 and glucose was present in the medium. Whereas, the value of the growth rate index was 2-fold lower in the bacterial culture and 48% of the growth reduction was obtained (Fig. 1). Different values have been noted for the cultures with sucrose providing carbon. The value of the growth rate index decreased significantly in case of both the supernatant and the bacterial culture and amounted 33 and 38% respectively (Fig. 1).

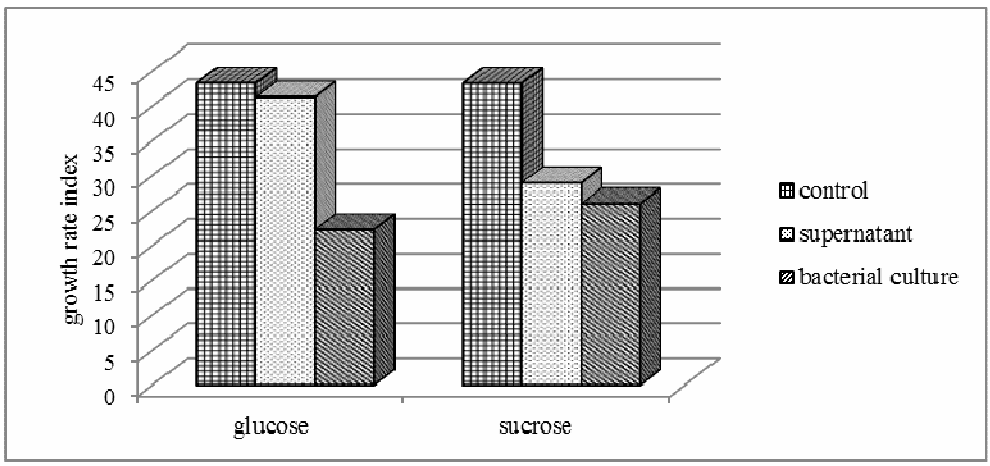

Fig. 1. The growth rate index for the trials with the inoculum at optical density 1

Similar relationship can be observed in trials with the inoculum at initial optical density equal 2.0. However, the inhibitory properties of *B. subtilis* are lower despite the 2-fold increase in the amount of the inoculum. On the medium with glucose no significant differences have been noted between the control trial and test trials. The highest measured value of the growth inhibition index was noted on the medium with sucrose, where the inhibition amounted 21% for the supernatants and over 41% for the bacterial culture (Fig. 2).

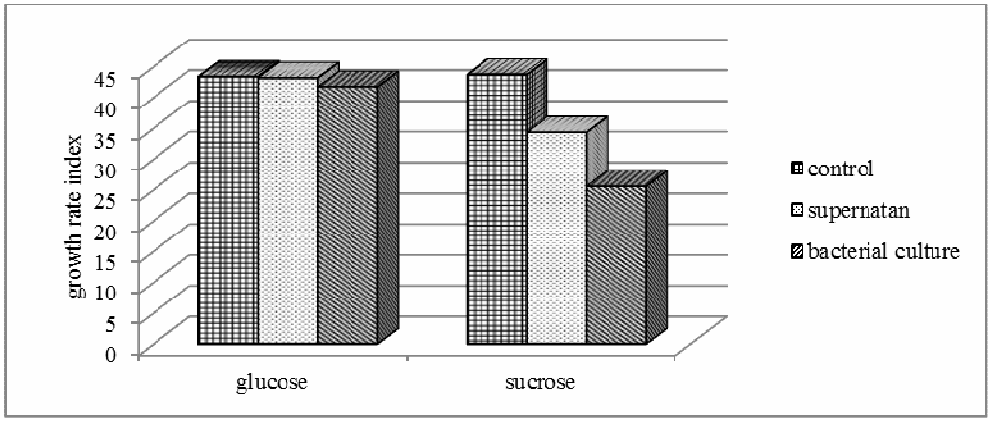

Fig. 2. The growth rate index for the trials with the inoculum at optical density 2

In the author's own research, beside the growth rate index enabling the assessment of mycelial growth in time, also the degree of the linear growth inhibition of *F. culmorum* has been take into account (Fig. 3). Higher values of the inhibition has been noted in the medium with sucrose. Regardless of the initial density of inoculum, the mycelial growth inhibition for both the supernatant and the bacterial culture has reached 20-38%, although the application of the latter proved to be more efficient. In the presence of glucose, 46% reduction has been obtained also in case of bacterial culture, with the initial density of inoculum equal 1 (Fig. 3).

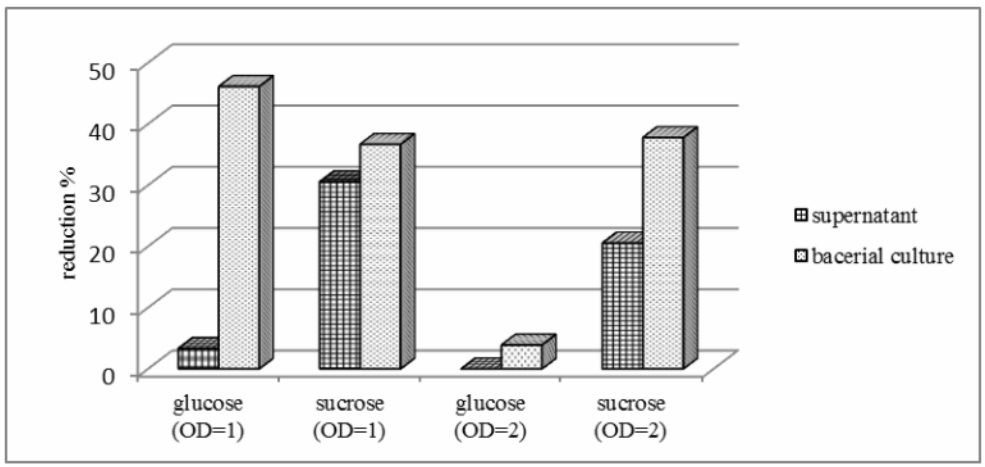

Fig. 3. *B. subtilis* affecting the linear growth of *F. culmorum.* (OD - *optical density*)

According to many authors, strains of *B. subtilis* are applied in the process of biological protection of plants due to their ability to produce antimicrobial chemical compounds, including predominantly peptides as well as a couple of non-peptidic

compounds such as polyketides, an aminosugar, and a phospholipid [11-13]. Antifungal activity of *Bacillus* spp. results from their ability to produce also other cyclic lipopeptides and the cell-wall degrading enzymes, of which the most valuable and important compounds belong to the group of peptide antibiotics. They are: surfactin, iturin and fengycin, which have a great potential of biotechnological and biopharmaceutical applications. Iturins and fengycins display a strong antifungal activity, however, surfactins are not fungitoxic by themselves but could synergistically impact the antifungal activity of other lipopeptides. Possibly lipopeptides affect the cell membrane of filamentous fungi to alter its permeability, resulting in release of cell contents [13-15]. According to other authors, *B. subtilis* might also act on pathogenic fungi by either producing antifungal substances or colonizing microsites faster than the surface fungi [16].

Conclusions

In conducted pilot research, strain *B. subtilis* showed the ability to affect the growth of *F. culmorum* in a varied way depending on the parameters under study. One, which played an important role in the metabolic activity of *B. subtilis*, was the source of carbon in the culturing medium. In most analysed trials, a significant growth reduction of *F. culmorum* has been noted in the presence of sucrose. In the trials, a higher degree of the growth reduction has been obtained with the inoculum at optical density 1.0. The results were also more favourable in case of bacterial cultures containing bacterial cells and their metabolites when compared with the results noted after the application of the supernatant.

A fungistatic activity of *B. subtilis* against *F. culmorum* has been proved in *in vitro* research and allows to conduct further studies at a wider range.

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UDZIAŁ ZEWNĄTRZKOMÓRKOWYCH METABOLITÓW BAKTERYJNYCH W ZAHAMOWANIU WZROSTU GRZYBÓW

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Abstrakt: W metodach biologicznej walki z fitopatogenami roślin stosuje się między innymi biopreparaty, które jako substancję czynną zawierają żywe komórki mikroorganizmów lub ich metabolity. Celem przeprowadzonych badań było określenie wpływ hodowli bakteryjnej *Bacillus subtilis* oraz jego supernatantu na wzrost grzybni *Fusarium culmorum*. Ocenę właściwości antagonistycznych *B. subtilis* przeprowadzono metodą hodowlano-płytkową. Badania przeprowadzono na dwóch różnych podłożach, zawierających glukozę lub sacharozę, uwzględniając w ten sposób wpływ źródła węgla na proces inhibicji. Hodowle prowadzono w temperaturze 25±2ºC przez 7 dni, dokonując co 2-3 dni pomiaru średnicy grzybni, a aktywność fungistatyczną *B. subtilis* określono w oparciu o indeks tempa wzrostu grzybni. Na podstawie uzyskanych wyników stwierdzono, iż w próbach kontrolnych indeks tempa wzrostu był na zbliżonym poziomie niezależnie od źródła węgla zawartego w podłożu. W próbach badanych nie odnotowano znaczącego zahamowania wzrostu grzybni w obecności glukozy. Wartości indeksu tempa wzrostu dla prób badanych były niższe jedynie o 0,2-1,7 jednostki w porównaniu do prób kontrolnych. Natomiast w hodowlach prowadzonych na podłożu zawierającym sacharozę uzyskano około 20% zahamowanie po zastosowaniu supernatantu i ponad 40% w obecności hodowli bakteryjnej *B. subtilis*.

Słowa kluczowe: *Bacillus subtilis*, *Fusarium culmorum*, aktywność przeciwgrzybowa