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# ANTIFUNGAL EFFICACY OF *Bacillus amyloliquefaciens* AGAINST *Alternaria* sp.

## SKUTECZNOŚĆ PRZECIWGRZYBOWA *Bacillus amyloliquefaciens* WOBEC *Alternaria* sp.

**Abstract:** The aim of the research was to assess a potential biological activity of *B. amyloliquefaciens* against *Alternaria* sp. In the conducted studies taken into account three factors: the bacterial cell density, the presence of the bacterial cells or the cell-free supernatant and the composition of the medium. The antagonistic properties were assayed with a dual culture plate method on PDA and Czapek media. The culturing process was conducted at 25 ±2°C for 14 days. The influence of the metabolites produced by *B. amyloliquefaciens* on the growth of *Alternaria* sp. was evaluate on the basis the growth rate index. Conducted studies have shown that the linear growth of *Alternaria* sp. was 6-fold and 5-fold slower after application of bacterial culture with the optical density equal 2.0, respectively on PDA and Czapek media, compared to the control tests. Furthermore, it was a slightly stronger inhibitory properties of *B. amyloliquefaciens* on PDA medium, wherein the carbon source was glucose than on Czapek medium where the carbon source was sucrose. This difference was about 1-3%. The linear growth of the fungus was inhibited more strongly by bacterial culture (approximately 2-3%), compared to the cell-free supernatant, regardless of the density of the cells and type of medium. Taking into account all the analyzed factors, the application of bacterial culture with a density of 2.0 on PDA medium, resulted in the slowest the growth of this fungus, approximately 83% compared with the control tests. *B. amyloliquefaciens* may find a wide range of application, in the process of plant protection against diseases caused by *Alternaria* sp.

**Keywords:** *Bacillus amyloliquefaciens*, *Alternaria* sp., fungistatic activity

## Introduction

Fungi of the genus *Alternaria* are common in many parts of the world, they are cosmopolitan organisms and can be found in soil, plants, food and indoor air. Some of them are saprophytes, and others are pathogens of various crop plants, ornamentals, fruit and vegetables. They affected the various stages of the plant development resulting in, among others, seedling blight, leaf spot, root rot and diseases of various plants called alternariosis, contributing to significant economic losses. They also contribute to a significant reduction in seed yield and the spoilage of agricultural products during storage and during transport. [1-3]. Furthermore, *Alternaria* spp. produces dangerous to plants, animals and humans secondary metabolites with toxic properties (mycotoxins), among others, radicin (RAD), *epi*-radicinol (*epi*-ROH) by *A. radicina* and tenuazonic acid (TeA), altertoxin I, II, III (ATX I, II, III), alternariol (AOH) by *A. alternata* [1, 2, 4, 5]. One of most effective measures to control the disease caused by *Alternaria* sp. is effective application of fungicides [1].

More and more often in the plant crops strives to limit the use of pesticides because they are expensive, harmful to the environment and sometimes ineffective due to the immunization of pathogens. Intensive studies are being conducted on the possibility the introduction of new and safer methods to limiting the growth and development phytopathogens. Therefore, from year to year growing an interest in biological preparations

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using the non-pathogenic soil microorganisms of the genera *Bacillus* and *Psudomonas*. These microorganisms are known as PGPR (Plant Growth Promoting Rhizobacteria) that are able exert a beneficial direct or indirect effect on growth and development of plant. The direct impact of microorganisms is associated with enrichment of soil in nutrients and increasing their bioavailability for plants, synthesis the phytohormones or vitamins. Indirect effects result mainly from the improvement in the plant health by inhibiting the growth of pathogens and induction of resistance of plants to disease. The positive impact of the bacteria is possible due to the production by them the biological active metabolites *eg* antibiotics or cell wall degrading enzymes. Therefore, *Bacillus* spp. strains may be an alternative to the use of chemical substances, as according to the European Union Directives still seeks systematically to limit the use of chemicals in the environment [6, 7]. The aim of presented studies was to determine the biotic interaction of *Bacillus amyloliquefaciens* with the *Alternaria* sp.

## Materials and methods

In this study the ability of the secondary metabolites produced by *Bacillus amyloliquefaciens* in limiting the growth of *Alternaria* sp. has been determined. The antagonistic properties of metabolites were assayed with a dual culture plate method on PDA medium consisting of [g/dm$^3$]: glucose 20.0, potato extract 4.0, agar 15.0 and on Czapek medium consisting of [g/dm$^3$]: sucrose 30.0, $MgSO_4 \cdot 7H_2O$ 0.5, $KH_2PO_4$ 1.0, KCl 0.5, $NaNO_3$ 3.0, $Fe_2(SO_4)_3 \cdot 7H_2O$ 0,01, agar 15.0.

Fungal mycelial-disks (diameter of 10 mm) obtained from growing cultures of test fungal isolates were placed on the centre of this media inoculated with 0.5 cm$^3$ bacterial cells (BC) and cell-free supernatant (CFS) obtained from 24-hour culture of *B. amyloliquefaciens* at a different bacterial cell density (OD = 1.0 and OD = 2.0). In the control plates both of bacterial supernatants and bacterial culture were replaced with sterile broth medium. All plates were incubated at 25 ±2°C for 14 days. The colony diameters of the fungal pathogens in the test and the control plates were measured every 2-3 days until the mycelium of *Alternaria* sp. in the control plate, reached the edge of the plate. Each experiment was run in triplicate. The antagonistic activity of this bacterium was estimated as the growth rate index (*T*), calculated according to the formula below [8]:

$$T = \frac{A}{D} + \frac{b_1}{d_1} + ... + \frac{b_x}{d_x}$$

where *A* is the mean from colony measurement [mm], *D* is the experiment duration (number of days), $b_1 ... b_x$ is the increase a colony diameter from lasted measurement, $d_1 ... d_x$ is the number days from lasted measurement.

During the evaluation of the results was designated the percentage reduction of the growth rate index in the treated plate versus the growth rate index in the control plate.

## Results and discussion

Several strains belonging to the genus *Bacillus eg B. amyloliquefaciens* and *B. subtilis* are able to excrete one or more compounds, among them bioactive non-volatile cyclic lipopeptides with fungistatic properties and the cell wall degrading enzymes. *Bacillus*

*amyloliquefaciens* strains were reported effective for the biocontrol of multiple plant diseases caused by soilborne or post-harvest pathogens [9-11].

Therefore, the antifungal activity of *B. amyloliquefaciens* on 2 different media was evaluated toward tested *Alternaria* sp. Due to the fact that many of the factors determine the activity of the microorganisms in the conducted studies taken into account three: the bacterial cell density (OD), the presence of the cells or the cell-free supernatant and the composition of the medium. Conducted studies have shown differences in fungistatic activity of *B. amyloliquefaciens*, depending on the parameters analyzed.

It was observed that the linear growth of the mycelium of *Alternaria* sp. on the PDA medium was inhibited most efficient by adding both bacterial cells (BC) and cell-free supernatant (CFS) to the growth medium compared to the control test (Fig. 1). The value of the growth rate index for bacterial cells was similar when the optical density of the inoculum was 1.0 and 2.0 (7.31 and 7.54 respectively). The highest measured value (8.27) was recorded after application of the cell-free supernatant at optical density equal 1.0. The linear growth of *Alternaria* sp. was 6-fold slower on this medium compared to the control test.

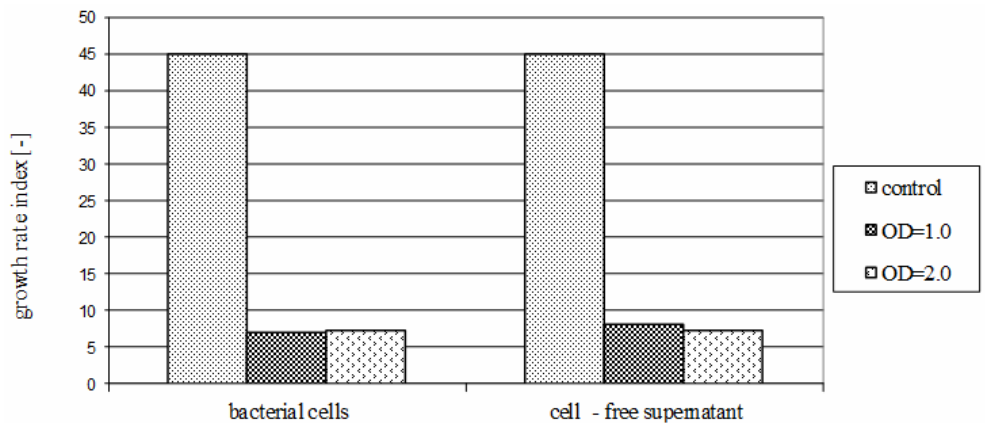


Fig. 1. Influence of *Bacillus amyloliquefaciens* on the growth rate index of *Alternaria* sp. on PDA medium (OD - optical density)

Similar activity of the metabolites of *B. amyloliquefaciens* against *Alternaria* sp. were observed on the Czapek medium with the sucrose. However, the inhibitory efficacy of this bacterium was slightly lower, when the cell-free supernatants were obtained from both inoculum with an initial optical density equal 1.0 and 2.0, compared to used the bacterial cells. The value of the growth rate index of *Alternaria* sp. amounted from 6.82 to 7.54 and was still 5-fold lower than the control test (41.07) (Fig. 2).

On both media, the difference in the inhibitory action of metabolites between the most and least acting culture of *B. amyloliquefaciens* amounted about 1.0-1.5 units.

The highest measured value of the growth inhibition was noted for the PDA medium, wherein as the carbon source was glucose than for the Czapek medium where as the carbon source was sucrose (Fig. 3).

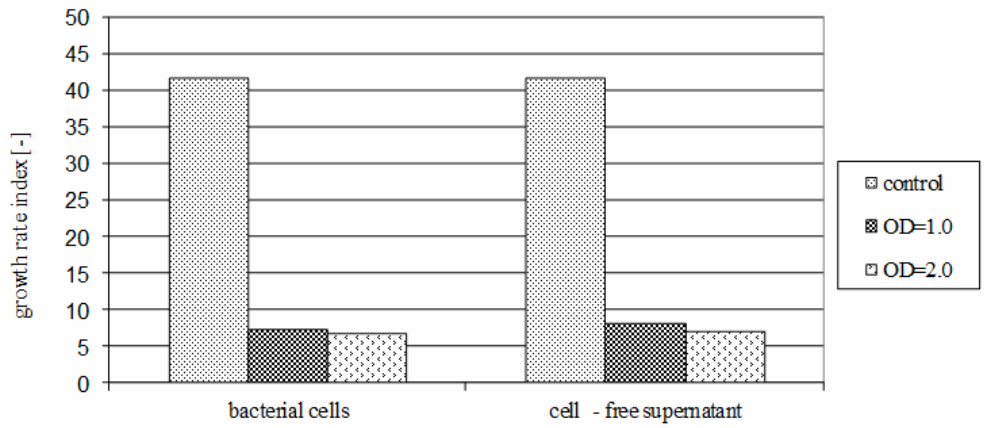


Fig. 2. Influence of *Bacillus amyloliquefaciens* on the growth rate index of *Alternaria* sp. on Czapek medium (OD - optical density)

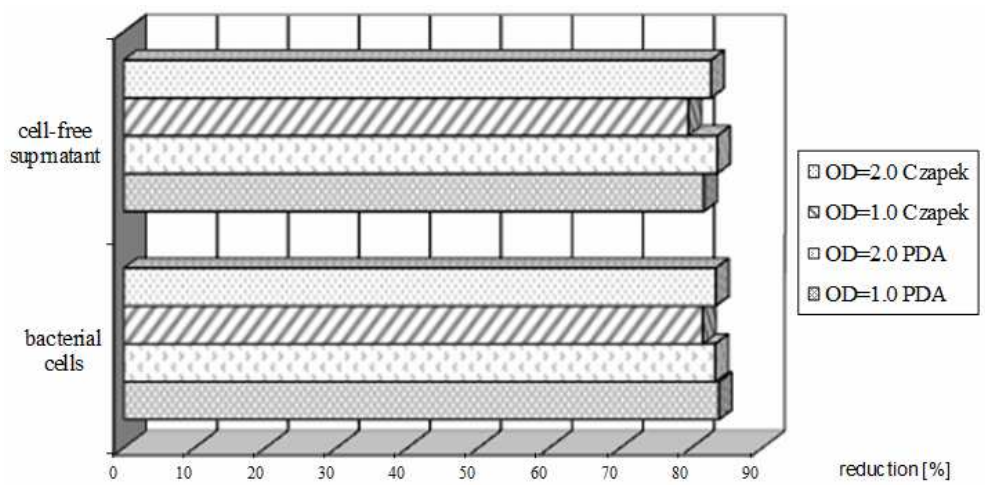


Fig. 3. Inhibition effect of *B. amyloliquefaciens* on mycelial growth of *Alternaria* sp.

The linear growth of the mycelium was inhibited most actively when the PDA medium was supplemented with the bacterial cells (optical density equal 1.0) and with the cell-free supernatant (optical density equal 2.0). The amounted obtained were: 83.85 and 83.60% respectively. Slightly lower activity of this bacterium was observed on the Czapek medium but it was still very high compared to the PDA medium. The inhibition of the growth of *Alternaria* sp. in case of both the bacterial cells and the cell-free supernatant for inoculum at initial optical density equal 2.0 ranged from 82.73 to 83.39 percent respectively. Taking into account all the analyzed factors, the application of bacterial culture with a density of 2.0 on PDA medium, resulted in the strongest inhibitory effect, by approximately 83%

compared with the control test. This might be due to production of metabolites secreted by this strain, diffused and dissolved into the culture media.

The possibility of controlling the soil-borne pathogens by introducing specific antagonistic bacteria to infected soil has been extensively investigated during the last decades. Extensive research has demonstrated that *Bacillus* spp. could have an important role in agriculture and horticulture in improving crop productivity. They have the capacity to colonize the rhizosphere and phyllosphere and to produce a broad spectrum of bioactive metabolites with antagonistic activity, among which are: cyclic lipopeptides (CLPs) of the surfactin, iturin and fengicin families [6, 7, 9, 12, 13]. Moreover, these bacteria are able to exhibit hyperparasitic activity, attacking pathogens by excretion lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, protein, cellulose [7, 14]. Fungistatic activity is conditioned by many factors, primarily depends on the biological properties of the strain, susceptibility of the fungus and the number of the antagonist compared to the population of pathogen [15].

It is well known that production of most of antibiotics and its antagonistic properties are dependent on the composition of medium where the microorganisms is grown [16-18]. Fructose, sucrose and mannitol in the concentration 1% were better as carbon sources than glucose or glycerol for iturin A production [17]. Islam et al [18] reported that mannitol (1%) was selected as the optimum sources of carbon, for use in production of antibiotic substances by *B. subtilis* C9. The antifungal substance has been widely reported in *B. amyloliquefaciens* cultures [10]. For example, *B. amyloiquefaciens* FZB 42 is known to produce of both bacillomycin and fengycin which can suppress *F. oxysporium* [9]. Mitoi et al [19] observed drastic modification of cell structure, which can indicate a lytic effect of antagonistic bacteria against fungi, like degradation of membrane system, apparition of protuberance, formation of fibrous layer on the outer surface of the fungi cells, plasmalemmal detachment, constriction of cytoplasm and degradation of membrane system. The antifungal activity of iturin lipopeptides is related to their interaction with cytoplasmic membrane of terget cells leading to an increase in $K^+$ permeability [19, 20].

In addition, these substances not only inhibit the linear growth of colonies, but also inhibit the formation of spores or sclerotia. Inhibition of mycelial growth and production of morphological elements may significantly reduce the survival of pathogens in the soil [21]. *Bacillus amyloliquefaciens* strains were reported effective for the biocontrol of multiple plant diseases caused by soilborne or post-harvest pathogens. These bacteria can strongly inhibit the growth of many plant pathogenic fungi *eg Alternaria* sp., *Fusarium* spp., *Glomerella cingulata*, *Phytophthora drechsleri*, *Botritis cinerea* [15, 18, 19, 22]. According to Saideelfeen et al [23] the twenty seven of forty five *Bacillus* isolates displayed antagonism against *A. alternata* in-vitro, due to the production of antimicrobial compounds. Since the *Alternaria* species infect crops of economic importance, there is a strong need to effectively control for this pathogen.

Therefore *B. amyloliquefaciens* may be an alternative to the use of chemicals, as according to the directives of the European Union still seeks systematically to limit the use of chemicals in the environment.

## Conclusions

The growth of *Alternaria* sp. was 6-fold and 5-fold slower after application of bacterial culture with a density of 2.0, respectively on PDA and Czapek media, compared to the control test. Furthermore, it was a slightly stronger inhibitory activity of *B. amyloliquefaciens* on PDA medium, wherein the carbon source was glucose than on Czapek medium where the carbon source was sucrose. This difference was about 1-2%. The linear growth of the fungus was inhibited more strongly by bacterial cells (BC) (approximately 1-3%), compared to the cell-free supernatants (CFS), regardless of the density of the cells and type of medium. Taking into account all the analyzed factors, the application of bacterial culture with a density of 2.0 on PDA medium, resulted in the slowest the growth of this fungus, approximately 83% compared with the control trial. Results obtained in this study suggested that *B. amyloliquefaciens* strain produced either a broad-spectrum antimicrobial compound or several bioactive compounds with different activities making it a potential candidate for use in the biocontrol of fungal plant diseases of agricultural importance.

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# SKUTECZNOŚĆ PRZECIWGRZYBOWA *Bacillus amyloliquefaciens* WOBEC *Alternaria* sp.

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**Abstrakt:** Celem przeprowadzonych badań była ocena właściwości przeciwgrzybowych *B. amyloliquefaciens* wobec *Alternaria* sp. W badaniach uwzględniono trzy parametry: gęstość komórek bakteryjnych, rodzaj podłoża oraz wpływ hodowli zawierającej komórki bakterii lub płyn pohodowlany. Ocenę właściwości antagonistycznych metabolitów bakteryjnych przeprowadzono metodą hodowlano-płytkową z zastosowaniem podłoży Czapka i PDA. Hodowlę prowadzono w temp. 25 ±2°C przez 14 dni. Na podstawie indeksu tempa wzrostu określono aktywność fungistatyczną *B. amyloliquefaciens.* Badania wykazały różnice w aktywności metabolicznej bakterii w zależności od analizowanych parametrów. Liniowy wzrost *Alternaria* sp. był prawie 6-kronie słabszy na podłożu PDA i 5-krotnie słabszy na podłożu Czapka po zastosowaniu hodowli bakterii o gęstości $E$ = 2,0. Ponadto nieznacznie silniejsze właściwości hamujące *B. amyloliquefaciens* odnotowano na pożywce PDA, w której źródłem węgla była glukoza, niż na pożywce Czapka zawierającej sacharozę. Różnica ta wynosiła około 1-3%. Liniowy wzrost grzyba był również silniej hamowany o ok. 2-3% po zastosowaniu hodowli bakteryjnej w porównaniu do supernatantu, niezależnie od gęstości komórek i rodzaju pożywki. Biorąc pod uwagę wszystkie analizowane czynniki, zastosowanie hodowli bakteryjnej o gęstości 2,0 i pożywki PDA spowodowało zmniejszenie tempa wzrostu tego grzyba o około 83% w porównaniu do próby kontrolnej. *B. amyloliquefaciens* może znaleźć szerokie zastosowanie w procesie ochrony roślin przed chorobami wywołanymi przez *Alternaria* sp.

**Słowa kluczowe:** *Bacillus amyloliquefaciens*, *Alternaria* sp., aktywność przeciwgrzybowa