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Original article

ISOLATION AND CHARACTERIZATION OF BACILLUS BACTERIA WITH ANTAGONISTIC PROPERTIES AGAINST THE PATHOGENS *ALTERNARIA ALTERNATA* AND *FUSARIUM GRAMINEARUM*

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ABSTRACT

Fungal diseases cause significant damage to crop production, making the protection of crops from harmful organisms essential for ensuring food security. Today, developing biological methods for crop protection is increasingly relevant. In this context, introducing preparations with biocidal action into agricultural practices is of great interest. These preparations should include bacterial strains with antagonistic properties against phytopathogenic microorganisms. The antagonistic properties of *Bacillus* bacteria are particularly noteworthy, as their metabolites can degrade mycotoxins, potentially reducing their toxicity. The search for *Bacillus* strains suitable for creating effective biofungicides against *Fusarium* and *Alternaria* diseases in plants holds promising prospects.

Key words: *Bacillus, Alternaria* blight, *Fusarium* blight, antagonism, phytopathogen.

1. INTRODUCTION

Microorganisms that cause plant diseases result in significant losses in agricultural crop yields. Among these, phytopathogenic fungi, particularly those from the genera *Fusarium* and *Alternaria*, are leading culprits [1]. The damage caused by these fungi includes reduced leaf photosynthetic surface, decreased yields, and harm to fruits and seeds. Additionally, both *Fusarium* and *Alternaria* produce mycotoxins, which pose serious risks to humans and animals when they contaminate food and feed [2].

In recent decades, natural antagonists of phytopathogenic fungi have been explored as alternatives or supplements to traditional fungicides. These non-pathogenic microorganisms, which inhabit plant residues in the soil, the rhizosphere, phyllosphere, or endophytically within plants, can compete with phytopathogens, suppress their growth, and positively affect plant health [3, 4].

Bacteria of the genus *Bacillus* have shown great agricultural potential due to their production of lipopeptides with high activity against phytopathogens [5]. The antifungal activity of *Bacillus* species is mainly attributed to three families of cyclic lipopeptides: surfactin, iturin, and fengycin. These molecules possess amphiphilic properties that disrupt biological membrane structures. Their antimicrobial activity extends to bacteria, fungi, oomycetes, and viruses. Recent studies also highlight these compounds' ability to stimulate plant defense mechanisms and biofilm formation, which are crucial for the successful biocontrol of phytopathogens [6].

Worldwide, antifungal biopreparations are being developed based on beneficial strains of *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. megaterium*, *B. velezensis*, *B. cereus*, and *B. thuringiensis* [7, 8]. Endophytic bacteria, which colonize niches similar to those of plant pathogens, are preferred candidates for biocontrol. Selecting local bacterial strains from wheat grain and various plant parts is advantageous, as these strains are already adapted to their environment [9]. When thoroughly studied, *Bacillus* bacteria have the potential to replace many of the chemical fungicides currently used against *F. graminearum* and *A. alternata*, which negatively impact the environment [10].

2. MATERIALS AND METHODS

2.1 Isolation of Bacillus Genus Bacteria from Seed Material

The seed material complies with GOST 12044-93, «Seeds of Agricultural Crops: Methods for Determining Disease Contamination.» Wheat seeds are first washed under running water for 1-2 hours, then disinfected with 96% alcohol for 1-2 minutes. After disinfection, the seeds are rinsed with sterile water and dried between layers of sterile filter paper. Five seeds are placed in a Petri dish with a nutrient medium and incubated in a thermostat at 25-27°C for germination, which typically lasts 3-7 days. Bacterial colonies are then stained using the Gram stain method.

2.2 DNA extraction and molecular characterization

The genomic DNA of separate colonies of the microorganisms was isolated using the bacterial DNA isolation kit (Biosilica). The isolation was performed according to the kit instructions. The quality of genomic DNA was monitored by electrophoresis on a 1 % agarose gel. Electrophoresis was carried out in a Max Fill HU10 horizontal electrophoresis chamber and a Consort EV 243 current source. 1× TAE buffer was used as an electrode buffer. The 16S rRNA was amplified using the primer pair: forward *16SrRNA-8F* (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse *16SrRNA-806R* (5'-GGACTACCAGGGTATCTAAT-3') (Sigma-Aldrich, USA). For all used primers, we prepared 20 **µl** mixture that contained 25 ng of each target DNA. The mixture also contained Taq DNA Polymerase (Fermentas), 0.2 mM of each dNTP, 1× PCR buffer, 2.5 mM $MgCl_2$, and 10 pmol of each primer. The

PCR program was run on a Master cycler Gradient, (Eppendorf) amplificator.

2.3 PCR samples purification

PCR samples were purified from oligonucleotide residues by dephosphorylation using alkaline phosphatase (SAP - shrimp alkaline phosphatase) and endonuclease. A mixture was prepared in a total volume of 10 µl for each sample - dH_2O - 7.25 µL, $10^{\times}$ PCR Buffer - 1.0 µl, $MgCl_2$ - 1.0 µl, SAP (5 mM) - 2.5 µl, Exonuclease I (5 units/µL) - 0.125 µl. The resulting mixture was added to each PCR product, placed in a thermal cycler under the following conditions: 37°C - 30 min, 85°C - 15 min, 4°C - ∞. Sample preparation for sequencing carried out by precipitation with an alcohol-acetate mixture.

2.4 DNA sequencing

The components of a standard set of reagents for the sequencing reaction were prepared in a 0.2-ml thin-walled thermocycler tube. A standard set of reagents for cyclic sequencing using *CEQ WellRED* terminator dyes (partially mixed). The following thermal cycle program was chosen: 96°C - 20 sec, 50°C - 20 sec, 60°C - 4 min for 30 cycles and followed by aging at 4°C. The sequencing was done by using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the sequence was deposited in GenBank. These sequences were compared with other sequences in the GenBank by using the BLAST analysis. The phylogenetic analysis was carried out with MEGA 6 software.

2.5 Study of Antagonistic Properties of Bacteria

Testing of antagonistic bacteria of the genus *Bacillus* against the fungi *Alternaria alternata* and *Fusarium graminearum* is conducted as follows: Sterile filter disks are dipped in a suspension of bacterial cells prepared in a physiological solution. These disks are then placed on three-day-old fungal colonies, with a disk soaked in physiological solution placed on the fourth side as a negative control. Observations of changes in the growth of both fungal and bacterial colonies are made over a period of up to 7 days.

3. RESULTS

During the experiment, the seed material is incubated on a nutrient medium to isolate antagonist bacteria. Four colonies of antagonist bacteria of the genus *Bacillus* were identified. For primary identification, Gram staining was performed, and the bacteria were viewed under a microscope (Figure 1).

As shown in Figure 1, the colonies of antagonist bacteria initially exhibited activity against other cultures. The colony growth pattern is characteristic of *Bacillus* species. Microscopy confirmed the presence of gram-positive, rod-shaped bacteria that form intracellular spores, typical of the *Bacillus* genus. To further confirm the cultural-morphological identification, DNA was isolated from the bacteria for molecular genetic analysis to determine the species composition. Amplification of genomic DNA using species-specific primers produced bands approximately 800-900 bp in size (Figure 2).

The PCR products of the bacterial strains were sequenced, and the nucleotide sequences were deposited in the NCBI GenBank database: 1) *Bacillus amyloliquefaciens* PQ222680, 2) *Bacillus cereus* PQ223301, 3) *Bacillus subtilis* PQ223368, and 4) *Bacillus thuringiensis* PQ223302. The test for antagonistic properties against *Alternaria alternata* and *Fusarium graminearum* was conducted on three-day-old fungal colonies. Discs impregnated with a bacterial cell suspension were placed on three sides of the colonies. The experimental results are presented in Figures 3 and 4.

In Figure 3, all *Bacillus* genus antagonist bacteria exhibit high antagonistic properties against *Alternaria alternata*. The results of their antagonistic properties against *Fusarium graminearum* are shown in Figure 4.

Figure 4 shows that all *Bacillus* genus bacteria-antagonists exhibit strong antagonistic properties against *Fusarium graminearum*. Thus, the data indicate that *Bacillus* bacteria-antagonists effectively suppress the growth of *Alternaria alternata* and *Fusarium graminearum*. *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus thuringiensis* isolates are recommended as biological fungicides against these phytopathogenic fungi.

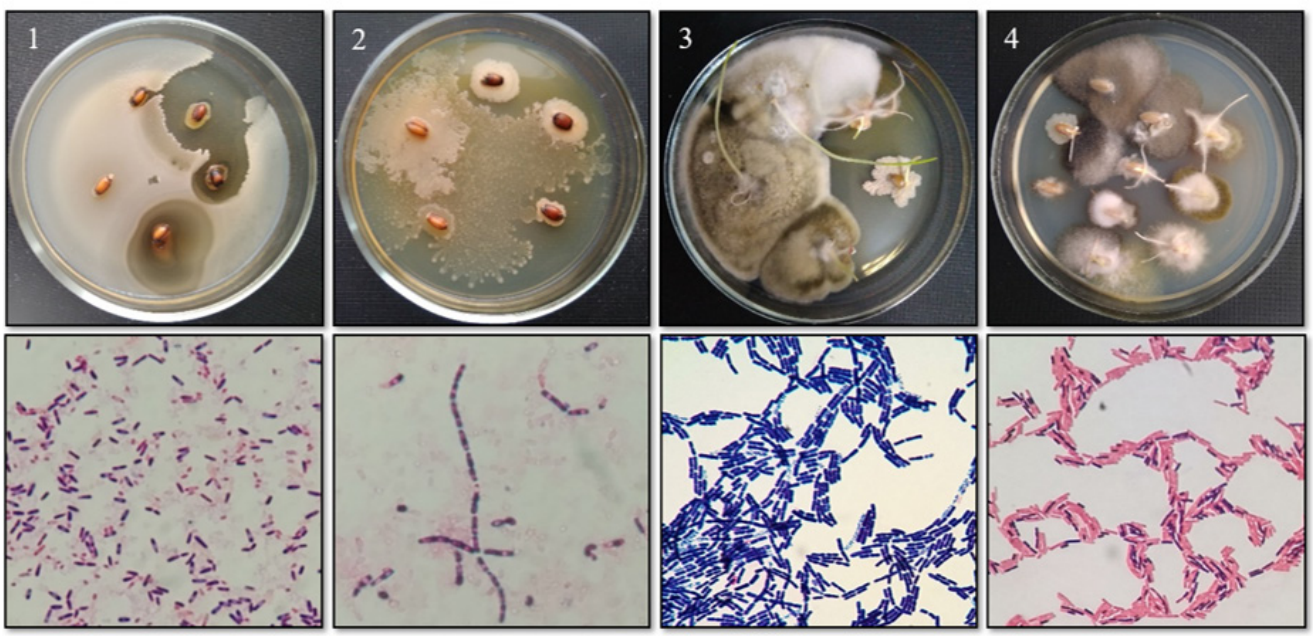

Figure 1 – Colonies of antagonist bacteria and their microscopic view (×100).

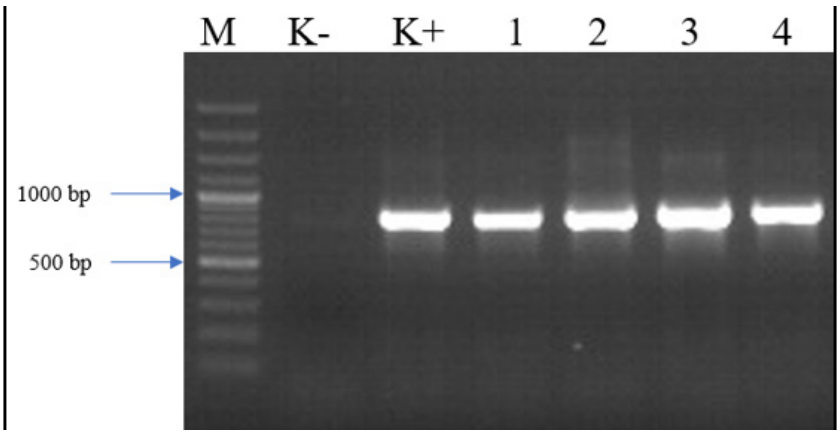

Figure 2 – Electrophoretic analysis of PCR products obtained with DNA of the bacterial strains: M – DNA ladder (100 bp); K- – negative control; K+ – positive control; 1-4 – DNA Bacillus spp.

4. Discussion

The increasing anthropogenic load on the environment and the need for food safety highlight the importance of advancing research in biological plant protection, particularly against toxinogenic fungi of the genera *Fusarium* and *Alternaria*. These fungi negatively impact both the quality and quantity of harvests and contribute to mycotoxin contamination [11].

Continuous monitoring of the phytosanitary situation in agricultural crops, rigorous mycological and mycotoxicological control, and adherence to protective and agrotechnical measures are crucial. Utilizing developed methods for monitoring mycotoxin accumulation, including biological preparations that can block toxin biosynthesis or transform toxins into less harmful compounds, appears promising [12].

Bacillus species are promising candidates for effective biofungicides against *Fusarium* and *Alternaria* due to their spore formation, high viability, cost-effective cultivation, and the ability of specific strains to suppress fungal growth and reduce plant contamination with mycotoxins. These bacteria not only reduce toxin synthesis but may also biodegrade toxins through microbial metabolites [13, 14]. Additionally, bacterial metabolites can induce plant defense mechanisms and influence the composition of the pathogenic plant microbiome.

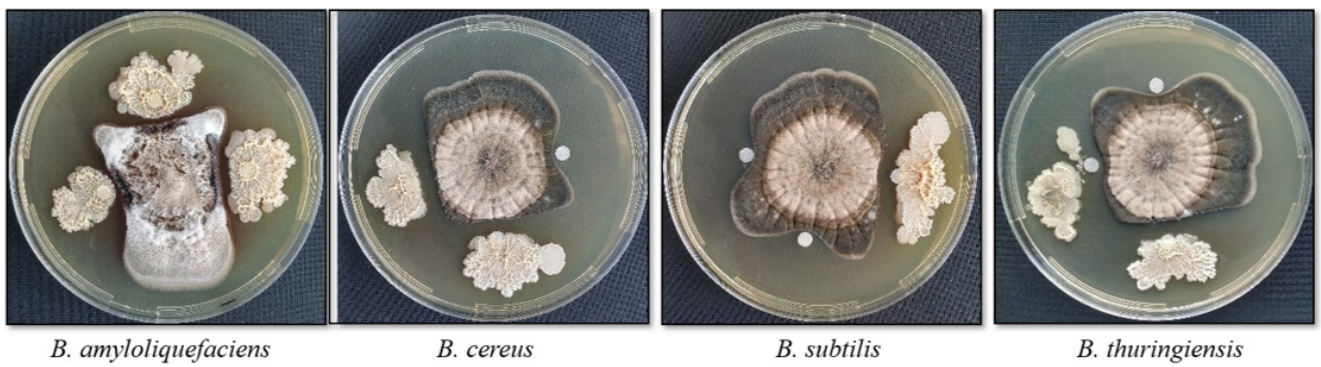

Figure 3 – Result of antagonistic properties against the fungus *Alternaria alternata*.

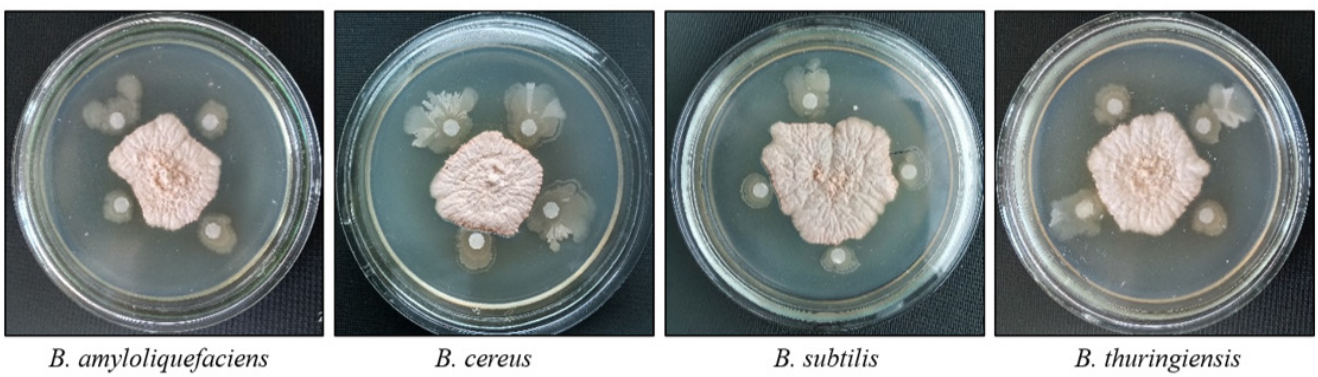

Figure 4 – Result of antagonistic properties against the fungus *Fusarium graminearum*.

CONCLUSION

As a result of the study, we isolated antagonist bacteria of the genus *Bacillus* from wheat seeds. Identification was conducted through cultural and morphological characteristics,

with molecular genetic confirmation establishing the species of the antagonist bacteria. The nucleotide sequences were deposited in the NCBI GenBank database with the following accession numbers: 1) *Bacillus amyloliquefaciens* PQ222680, 2) *Bacillus cereus* PQ223301, 3) *Bacillus subtilis* PQ223368, and 4) *Bacillus thuringiensis* PQ223302.

The antagonistic properties of these bacteria were tested against two major phytopathogenic fungi of cereal crops, *Alternaria alternata* and *Fusarium graminearum*, which reduce crop yields and compromise feed suitability for farm animals. Our findings demonstrate that *Bacillus* species possess significant antagonistic properties and are recommended for use as components in biological fungicides.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА БАКТЕРИЙ РОДА *BACILLUS* ОБЛАДАЮЩИХ АНТАГОНИСТИЧЕСКИМИ СВОЙСТВАМИ В ОТНОШЕНИИ ВОЗБУДИТЕЛЕЙ *ALTERNARIA ALTERNATA* AND *FUSARIUM GRAMINEARUM*

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АБСТРАКТ

Большой урон растениеводству наносят грибные заболевания. Защита урожая от вредных организмов является залогом обеспечения населения продовольствием. На сегодняшний день актуально получение биологических средств защиты урожая. В связи с этим представляет интерес внедрения в практику растениеводства препаратов, имеющих спектр биоцидного действия. К таким средствам следует отнести штаммы бактерий, проявляющие антагонистические свойства по отношению к фитопатогенным микроорганизмам. Раскрыты особенности проявления антагонистических свойств бактерий *Bacillus*, которые заключаются в способности метаболитов бактерий осуществлять деструкцию микотоксинов, что может способствовать снижению их токсичности. Обосновывается перспективность поиска штаммов бактерий рода *Bacillus,* пригодных для создания эффективных биофунгицидов против фузариозов и альтернариозов растений.

Ключевые слова: *Bacillus,* альтернариоз, фузариоз, антагонизм, фитопатоген.

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ALTERNARIA ALTERNATA ЖӘНЕ *FUSARIUM GRAMINEARUM* ҚОЗДЫРҒЫШТАРЫНА ҚАРСЫ АНТАГОНИСТІК ҚАСИЕТТЕРІ БАР *BACILLUS* ТЕКТЕС БАКТЕРИЯЛАРДЫҢ ОҚШАУЛАНУЫ ЖӘНЕ СИПАТТАМАСЫ

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АҢДАТПА

Саңырауқұлақ аурулары өсімдік шаруашылығына үлкен зиян келтіреді. Ауылшаруашылық дақылдарын зиянкестерден қорғау – халықты азық-түлікпен қамтамасыз етудің кілті. Бүгінгі таңда өсімдікті қорғаудың биологиялық құралдарын алу маңызды. Осыған байланысты өсімдік шаруашылығы тәжірибесіне биоцидтік әсер ету спектрі бар препараттарды енгізу қызығушылық тудырады. Бұл агенттерге фитопатогендік микроорганизмдерге қарсы антагонистік қасиет көрсететін бактерия штаммдары жатады. *Bacillus* бактерияларының антагонистік қасиеттерінің көріну ерекшеліктері анықталды, олар бактериялық метаболиттердің микотоксиндерді жою қабілетінен тұрады, бұл олардың уыттылығын төмендетуге көмектеседі. Өсімдіктердің фузариоз және альтернариоз ауруларына қарсы тиімді биофунгицидтер жасауға жарамды *Bacillus* тектес бактериялардың штаммдарын іздеу перспективалары негізделген.

Түйін сөздер: *Bacillus,* альтернариоз, фузариоз, антагонизм, фитопатоген.