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THE INFLUENCE OF DIFFERENT CLIMATIC TYPES ON THE NUMBER OF *Bacillus* spp. ISOLATED FROM SOIL IN NORTH MACEDONIA

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Abstract

Soil microorganisms play an important role in the biogeochemical processes of various elements vital to plant growth and animal life. Understanding and predicting the effects of climate change on soil microorganisms and their role in the ecosystem is a major challenge and provides an opportunity to focus research efforts on one of the most pressing problems facing our planet. *Bacillus* is a widely distributed genus with 347 species and 7 subspecies known to date. Members of this genus are capable of forming spores that are resistant to extreme heat, bactericidal agents and chemical disinfectants. Many *Bacillus* species are used in medicine and agriculture to produce antibiotics and also serve as probiotics in foods. Climate, as an abiotic factor, influences soil microorganisms by controlling the rate of soil formation and the chemical composition of the soil. Most soil microbiology studies focus on the diversity and abundance of soil microorganisms and on documenting the effects of environmental changes on these microorganisms. This new research trend can be applied to *Bacillus* spp. from soils in North Macedonia in the three climate types represented, mainly due to the climatic differences between them. This research focuses on the determination of soil geochemical parameters and microbiological analyses. A total of 36 strains of *Bacillus* spp. were isolated, 6 of which showed antimicrobial activity against certain test microorganisms. According to the results, it was also found that the diversity of *Bacillus* species changes depending on the soil microenvironment under the influence of different climatic conditions.

Key words: *soil bacteria, climate change, agriculture, antibiotics, probiotics*

INTRODUCTION

Climate change has major direct and indirect impacts on the composition and functions of terrestrial microbial communities. There has been a list of climate change impacts on terrestrial microbial communities. Among these are mortality and disturbance, direct and indirect effects on metabolic activity, biomass reduction (stimulation), diversity and composition leading to extinction/displacement, with negative or positive effects on physiology and greenhouse gas emissions (Tyagi et al., 2014). Microbial community structures tend to change with temperature increases, and respiration, fermentation, and methanogenesis processes are accelerated (Weiman, 2015). The risk of damage, disease, and death from heat waves, fires, severe storms, floods, natural disasters, extreme heat, poor air quality, drought, and the spread and development of disease are all part of the impact of climate change on biotic and abiotic components (Castro et al., 2010). Climate change is affected by bacteria, fungi, algae, and archaea (Fierer & Schimel, 2003). They contribute to global warming by increasing the rate of decomposition of organic matter, thereby increasing the amount of CO_2 in the atmosphere (Bardgett et al. 2008).

Microbial biodegradation of carbon in soil provides positive feedback for increasing the global temperatures. Microbial biomass and enzymes are efficient tools to enhance warming by breaking down carbon-containing organic matter and releasing hazardous substances into the environment while preventing climate change (Allison et al., 2010). The impact of temperature on enzyme activity and microbial physiological properties is direct (Friedlingstein et al., 2006). The rate at which soil microorganisms use carbon directly influences the soil response to climate change. When microorganisms are exposed to new extreme environmental conditions, their community composition, occurrence, and ecosystem function change, i.e., environmental changes or global warming/ climate disturbances affect microbial ecology, ecosystem structure, and function (Steinweg et al., 2008). In addition, significant changes in their functional genes and properties occur over time. Every biogeochemical cycle is subjected to these types of impacts/influences (Bradford et al., 2008). Generalizing the effects of climate change on soil microbial populations in different soil ecosystems is challenging because soils vary widely in terms of their biotic and abiotic properties. There are variations in biogeochemistry that control the types of microorganisms found in a given soil type, including pH (Fierer & Jackson, 2006) and salinity (Thompson et al., 2017). In addition, soil structure and moisture content influence the establishment of microbial niches (Schimel & Schaeffer, 2012) which in turn has a cascading effect on carbon and nutrient transformation. In order to understand better how climate change affects community species relations and metabolism (Cordero & Datta, 2016) the distribution and interconnectivity of soil microbial communities must be first examined. Considerable part of modern research in the field of soil microbial ecology concentrates on categorization of soil bacterial diversity and documentation of how soil bacterial communities change in response to certain environmental changes or disturbances (Fierer & Jackson, 2006; Thompson et al., 2017) and other research of similar nature could focus on the distribution of *Bacillus* spp. population in the soil of the Republic of North Macedonia, to which climatic variability is applied.

In this study, a strategy was used to determine the number of *Bacillus* spp. in different soil types from three different climatic regions in the Republic of North Macedonia: continental, submediterranean and alpine, in order to have a better understanding of the important species of *Bacillus* spp. in the soil in different climatic zones, and also about how the number of species of *Bacillus* spp. differs in various geographic and climatic areas.

MATERIAL AND METHODS

Sample collection

Samples were collected from different locations in the Republic of North Macedonia in three types of climatic zones. Soil samples were collected in May 2021 from three different locations in each climatic zone, ranging from the southwest to the southeast of the Republic of North Macedonia. The soil from the continental zone was collected from the village of Bistrica (Bitola), the soil from the alpine area from Mount Baba (Bitola) and the soil from the submediterranean area was taken from Vardarski Rid (Gevgelija). The selection took into account conservation, availability of the sample site and anthropogenic disturbances (proximity to a forest road, etc.). During sampling, the temperature in the respective area and the corresponding geographical information (coordinates, altitude) were recorded. The collection of the material in the field was carried out on experimental plots (wooden frames) of 1 m x 1 m. Samples were collected 15-20 cm below the surface. Soil was collected with a sterile spatula and transported in sterile bags in a refrigerator box with ice to the laboratory of the Department of Microbiology and Microbial Biotechnology at the Faculty of Natural Sciences and Mathematics, where a laboratory was accessed within 24 hours after the material was collected (Figure 1).

Figure 1. Soil sample collection.

Soil geochemical parameters

A total of three soil geochemical parameters were determined, including moisture content, soil pH, and organic carbon. After collecting the samples of soil, the moisture content was determined for each soil type. The samples were dried at 105 °C to a constant mass, after which the percent moisture loss in the samples was determined. The pH of the soil was determined using a pH meter. 20 g sample of each soil type was placed in a 100 ml glass beaker and 40 ml of distilled water was added. After stirring the suspension vigorously, it was left for 1 hour to enable the clay particles to precipitate, and the pH of the soil was measured. Organic carbon was determined by the Kotzman method, which is based on oxidation of organic carbon with $KMnO_4$ solution and retitration with oxalic acid (Figure 2).

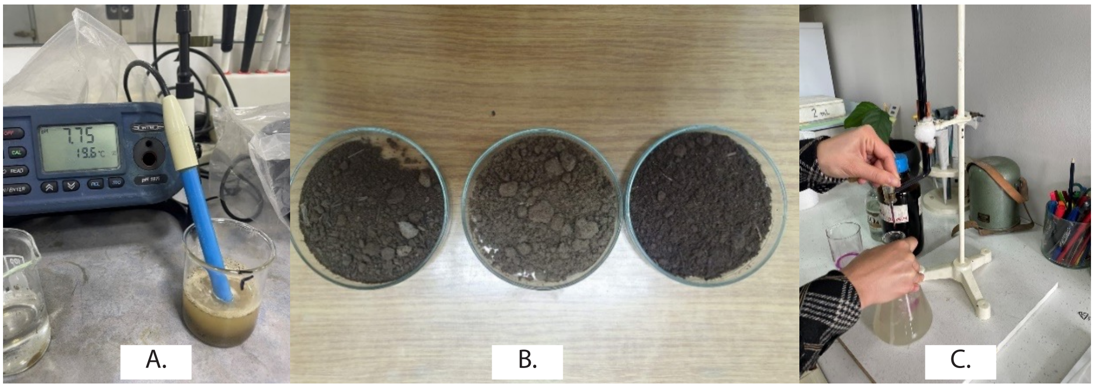

Figure 2. A. Determination of the pH value of the soil. B. Determination of moisture content. C. Determination of the organic carbon.

Determination of the number of *Bacillus* spp.

To determine the number of *Bacillus* spp. a dilution series of each soil type was prepared. For this purpose, 10 g of each soil sample was transferred to a sterile bottle containing 90 ml of sterile distilled water. After mixing the bottle vigorously, 5 ml of each was transferred to sterile empty test tubes, which were placed in a water bath at 70°C for 30 minutes. After expiration of this time, the sample was serially diluted. Each of the dilutions (10^{-1}-10^{-8}) was inoculated onto a nutrient agar plate (NA) using the pour plate method. The inoculated plates were incubated at 37 °C, and bacterial growth was monitored after 24 and 48 hours. Quantitative analyses of bacterial growth in the samples were carried out by determining the number of bacterial colonies expressed as CFU/g of the original sample. Qualitative analyses were determined by the morphological characteristics of the microorganisms of the genus *Bacillus*.

For this purpose, the macroscopic characteristics were then observed to describe the colony's morphological shape and colour. Before beginning the microscopic analysis, pure cultures were isolated from the previously inoculated plates and then microscopic slides were prepared using the Gram stain method. Microscopic observation was performed using a microscope with a x100 objective.

Determination of antimicrobial activity

The determination of the antimicrobial activity of the isolates against the test microorganisms was conducted using the agar well diffusion method. The test bacteria (*Salmonella enterica* ATCC 10708, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Listeria monocytogenes* ATCC 13393, *Bacillus subtillis* ATCC 6633, *Staphylococcus aureus* ATCC 25922) and test fungi (*Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231) used in this analysis are included in the microorganism collection of the Microbiology Laboratory at the Faculty of Natural Sciences and Mathematics, Skopje, North Macedonia. All test bacteria were inoculated in nutrient broth (NB) and incubated at 37°C for 24 hours, while the test fungi were inoculated in Sabourad-dextrose broth (SDB) and incubated at 25°C for 72 hours. Test microorganisms were inoculated onto each sterile nutrient agar (NA) Petri dish using a sterile swab, and then wells with a diameter of 6 mm were placed on the nutrient medium. Into these wells, cultures of *Bacillus* spp. were added which were allowed to pre-incubate at 37°C for 24 hours. The plates thus inoculated were incubated at 37°C for 24 hours, and then the diameter of the zone of inhibition was measured for each isolate.

RESULTS AND DISCUSSION

Sample collection

Geographic information, altitude, and temperature during soil collection are shown in Table 1.

Table 1. Geographic information, altitude, temperature for each soil type.

Location	Village Bistrica (Bitola)	Mount Baba (Bitola)	Vardarski Rid (Gevgelija)
Coordinates	N40°59'3"E21°22'40"	N41°02.3974'E21°13.0749'	N41°08'44"E22°21'30"
Altitude	587 m	1380 m	55 m
Temperature	20°C	11°C	24°C

Several factors influence the microbial community in the soil. One of them is climate, which is considered an important abiotic factor affecting other sub-factors that form the community of Bacillus spp. in soil regardless of climatic conditions and artificial changes. Climate change and global warming are causing a paradigm shift in soil microbial communities, making the study of microbial communities in different terrestrial ecosystems increasingly important. Several studies analysing seasonal variations in bacterial community profiles in alpine forest soils confirm that this approach is gaining importance in the study of soil microbial community composition at different climatic and geographic scales (Cordero & Datta, 2016). The Balkan Peninsula has very different climatic conditions that have a considerable impact on terrestrial ecosystems, changing the physiochemical properties of the soil and thus altering the profile of soil bacterial communities. This opens many opportunities to study the diversity of microbial communities in the soil of the Republic of North Macedonia in climatic and geographic dimensions that currently receive little attention.

Soil geochemical parameters

After determining the moisture content of the different soil types, the highest pH value was observed for the soil from the alpine area (Mount Baba, Bitola) (Table 2). When determining the pH value of the given soil types, the highest pH value (7.67) was found for the soil from the submediterranean area, followed by the soil from the continental area with a pH value of (7.53), while the lowest pH value (6.91) was determined for the soil from the alpine region.

Table 2. Moisture content for each soil type.

Location	Village Bistrica (Bitola)	Mount Baba (Bitola)	Vardarski Rid (Gevgelija)
% moisture	11,3%	19,08%	9,8%

According to the humus content, Gračanin divided the soils into: very low humus content (<1%), low humus content (1-3%), medium humus content (3-5%), high humus content (5-10%), very high humus content (>10%). From the results of the determination of organic carbon in the soils by the use of Kotzman method, the soil from the continental area (Bistrica village) is slightly rich in humus, the soil from the mountain area (Mount Baba) is strongly rich in humus, while the soil from the submediterranean area (Gevgelija) is extremely poor in humus (Table 3).

Table 3. Humus content in soil according to Kotzman's method.

Location	Village Bistrica (Bitola)	Mount Baba (Bitola)	Vardarski Rid (Gevgelija)
Humus (%)	2,097	5,017	0,77
	2,057	5,022	0,52

Soil parameters pH, moisture content and organic carbon showed significant differences between climatic zones. Moisture content was lower in the submediterranean region, but *Bacillus* spp. were more diverse than in other climatic zones, indicating a minor influence of moisture content on the survival of different species of *Bacillus* spp. This study shows the difference in the number of Bacillus spp. isolated from different soil types in different climates, despite the same growth conditions in the laboratory, which has never been reported in the literature. According to the results obtained in the determination of antimicrobial activity, several of these isolates have the potential for enzyme production as well as probiotic properties. Further research is needed to determine the relationship between the microbial population and other ecological characteristics in the area of Republic of North Macedonia, which could face catastrophic consequences as a result of urbanization and climate change. The extreme flexibility of the microbial genome has impacted microbial adaptation over time, allowing bacteria to rearrange and exchange genomic sequences and acquire advantageous traits in response to changing environmental conditions. Because members of soil biological communities vary in their physiology, growth rate, temperature sensitivity, and other environmental parameters (Delgado-Baquerizo et al., 2014) climate change is likely to alter their relative abundance and function.

Determination of the number of *Bacillus* spp.

The results of the determination of the number of *Bacillus* spp. for each soil type are presented in Table 4. According to the results, the highest number of *Bacillus* spp. was found in the mountain region and the lowest value for the number of *Bacillus* spp. in the soil was found in the submediterranean region, expressed as CFU/g dry soil.

Table 4. Number of *Bacillus* spp. for each type of soil

Location	Village Bistrica (Bitola)	Mount Baba (Bitola)	Vardarski Rid (Gevgelija)
Log10 CFUs/g	4,68	6,69	4,97

*CFUs (Colony Forming Units)

The bacteria isolates from soil samples from different climatic regions of the Republic of North Macedonia were isolated and sampled on nutrient agar plates. Morphological analysis showed that the isolates were Gram-positive, rod-shaped, sporulating bacteria (Figure 3).

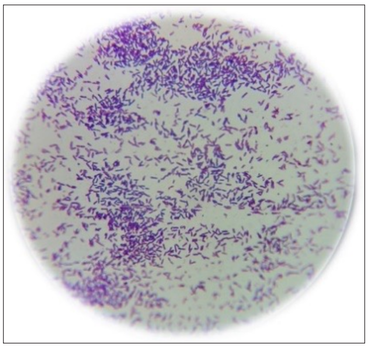

Figure 3. Microscopic characteristics of *Bacillus* spp. isolated from soil stained by the Gram method (x100).

Based on the data, although the genus *Bacillus* is ubiquitous in all major soil types of different climatic zones, the number of this genus's species varies considerably, indicating that climate has a significant influence on the microbial population of *Bacillus* spp. in different soils. The highest value for the number was found in the alpine region and the lowest value in the submediterranean region, which is associated with an increase in temperature and a decrease in altitude. It is probable that bacterial diversity and abundance decrease with increasing altitude and are affected by environmental and geological factors such as vegetation, temperature, and pH, which form a complex interaction. However, numerous studies have yet to uncover consistent changes in microbial populations along altitudinal gradients. Alterations in the relative abundance of organisms involved in regulating certain soil processes can directly affect the rate at which these are carried out, which in turn has implications for soil functioning and hence pollutant degradation rates. Because these processes can be carried out by a variety of species, phylogenetically broad process are generally more strongly linked to abiotic parameters (e.g., temperature, moisture) than to microbial community composition (Hooper et al., 2005).

Determination of antimicrobial activity

Screening of antimicrobial activity of the 6 isolates was performed against 9 test microorganisms. All isolates were found to have inhibitory activity against *Escherichia coli* ATCC 8739 and *Bacillus subtillis* ATCC 6633 (Table 5).

Table 5. Antimicrobial activity of *Bacillus* spp. isolated from soil against test bacteria

	Escherichia coli ATCC 8739	*Bacillus subtillis* ATCC 6633
Bacillus 1	/	8 mm
Bacillus 2	4 mm	4 mm
Bacillus 3	/	8 mm
Bacillus 4	2 mm	4 mm
Bacillus 5	4 mm	10 mm

Most of the isolates showed antimicrobial activity against the Gram-positive bacteria *Bacillus subtillis* ATCC 6633, 3 of the isolates showed antimicrobial activity against the Gram-negative bacteria *Escherichia coli* ATCC 8739. The first two isolates presented in Tables 4 and 5 were isolated from the sub-Mediterranean area, i.e., Gevgelija, while the others from the continental area, village Bistrica, Bitola. None of the isolates from the alpine area, Mount Baba, showed antimicrobial activity. The isolate *Bacillus* 5, isolated from the continental area, showed the greatest inhibitory effect on the test bacteria, i.e., the largest zone of inhibition for this isolate was observed against *Staphylococcus aureus* ATCC 25922 with a diameter of 10 mm (Figure 4).

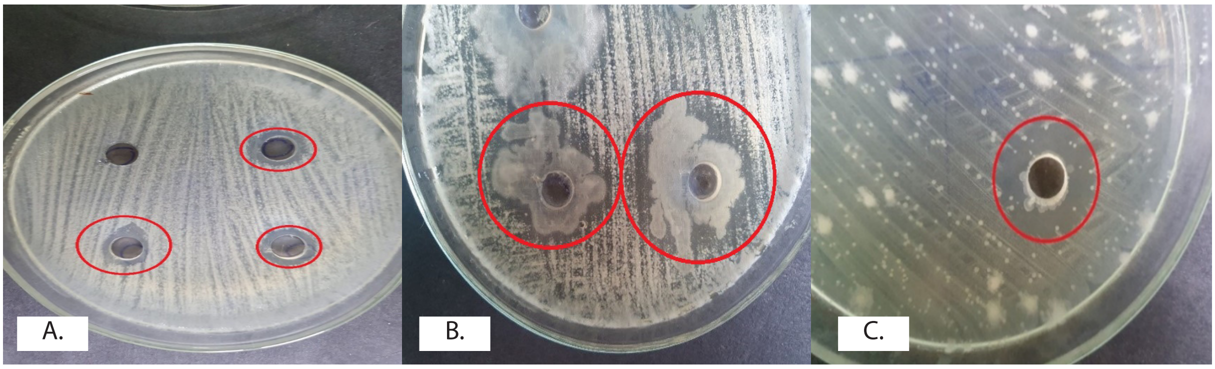

Figure 4. Antibacterial activity (zones of inhibition) of *Bacillus* spp. from soil against:
A. *Escherichia coli* ATCC 8739. B. *Bacillus subtillis* ATCC 6633. C. *Staphylococcus aureus* ATCC 25922

In terms of the antimicrobial activity against the test fungi, only two of the isolates showed a zone of inhibition against *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 (Table 6). Both isolates from the continental and submediterranean areas showed a zone of inhibition with a diameter of 12 mm against *Aspergillus niger* ATCC 16404 (Figure 5).

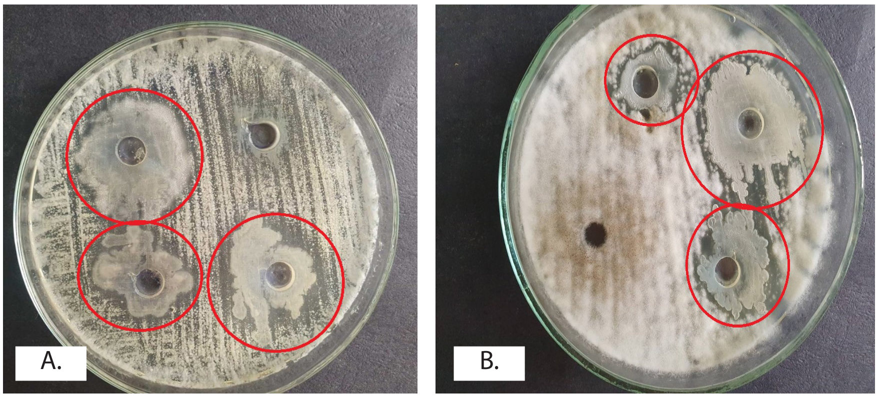

Figure 5. Antifungal activity (zones of inhibition) of *Bacillus* spp. from soil against:
A. *Candida albicans* ATCC 10231. B. *Aspergillus niger* ATCC 16404.

Table 6. Antimicrobial activity of *Bacillus* spp. isolated from soil against test fungi

	Test microorganisms	
	Candida albicans ATCC 10231	*Aspergillus niger* ATCC 16404
Bacillus spp.		
Bacillus 1	/	/
Bacillus 2	2 mm	12 mm
Bacillus 3	/	/
Bacillus 4	4 mm	12 mm
Bacillus 5	/	/

The ecosystem functions of soil microorganisms are crucial for maintaining soil carbon and providing nutrients to plants, and their value in preserving healthy soils for future generations cannot be overstated. There is an urgent need to better understand the effects of climate change on important biogeochemical processes carried out by soil microorganisms and to use this information to better predict climate and ultimately develop microbial strategies to counteract further climate warming and soil degradation (Classen et al., 2015). Although numerous approaches are being explored to use soil microorganisms to reduce the impacts of climate change, these would not be sufficient to compensate for the soil loss and greenhouse gas emissions that are occurring (Whitaker et al., 2014). To achieve this, details obtained from microbial population surveys need to be linked to ecosystem function resolution and various climate models, consistent with the recent “Warning to Humanity” reminder of the importance of the microbiota in maintaining ecosystem stability during climate change (Cavicchioli et al., 2019).

CONCLUDING REMARKS

Soil microbial populations are incredibly diverse and represent an important aspect of ecosystems that contribute to climate change through the breakdown of soil organic matter. Soil is the primary store of global terrestrial carbon, and fractional-level changes in the total soil carbon cycle can have significant consequences on atmospheric carbon dioxide density. As a result, the change in soil carbon is a key regulator of future climate in response to environmental change. In general, the consequences of global warming on the microbial community and subsequent decomposition processes are more pronounced in alpine, arctic and highland sites. However, compared to the diversity of macroorganisms, which has been studied for millennia, microbial population patterns have not been widely explored and remain poorly understood.

In the present study, some of the isolated *Bacillus* spp. showed antimicrobial activity against *Escherichia coli* ATCC 8739, *Bacillus subtillis* ATCC 6633 and *Staphylococcus aureus* ATCC 25922, these strains make excellent candidates for use as brand-new probiotic strains. Around the world, there has been a continuous search for innovative probiotics that are beneficial in industrial, agricultural, and medical settings. Potential probiotics must be able to pass the basic probiotics characteristics test, be able to withstand digestive factors especially those with low pH and high bile concentrations, produce antimicrobial substances, and adhere to the intestinal mucosa (Kesen & Olayinka, 2018). In addition, since these are preliminary results, to evaluate the long-term probiotic capability of these strains, it will be necessary to determine their antimicrobial activity against the most frequently used antibiotics as a control test and on a wider variety of pathogenic microorganisms, as well as on animals.

There are several ways that climate change affects our world, including changes in water temperature, pH, or oxygen content; a rise in desertification; wind patterns that are affected by land erosion; and prolonged dry spells in tropical forests. Each of these geographical shifts has an effect on the microbial communities in the area, which affects their metabolic capabilities and activities. The regional as well as the global climate can then be affected by these changes. According to the results, it was found that the diversity of *Bacillus* species changes depending on the soil microenvironment under the influence of different climatic conditions. There is still a lack of knowledge about how *Bacillus* assembles in the soil ecosystem, particularly throughout the year’s several seasons. The primary driver of the Bacillus community was temperature and humus content. Thus, the sum of our data offers a thorough understanding of the *Bacillus* community assembly in the soil ecosystem under different climatic conditions.

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ВЛИЈАНИЕТО НА РАЗЛИЧНИТЕ КЛИМАТСКИ ТИПОВИ ВО РС МАКЕДОНИЈА ВРЗ БРОЈНОСТА НА *Bacillus* spp. ИЗОЛИРАНИ ОД ПОЧВА

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Резиме

Почвените микроорганизми имаат главна улога во биогеохемиските процеси на различни елементи кои се од витално значење за растот на растенијата и животот на животните. Разбирањето на влијанието на климатските промени врз почвените микроорганизми и нивната улога во екосистемот претставуваат голем предизвик и можност за насочување на истражувачките напори кон еден од повеќето итни проблеми со кои се соочува нашата планета. *Bacillus* е широко распространет род кој брои 347 видови и 7 подвидови познати до денес. Членовите на овој род имаат способност да формираат спори кои се отпорни на екстремна топлина, бактерицидни агенси и хемиски средства за дезинфекција. Многу видови на *Bacillus* имаат широка примена во медицината и земјоделството поради производството на антибиотици, а се користат и како пробиотици во исхраната. Климата како абиотички фактор влијае врз почвените микроорганизми преку контролирање на брзината на формирање на почвата и нејзиниот хемиски состав. Поголемиот дел од истражувањата во областа на микробна екологија се фокусираат на бројноста на почвените микроорганизми, како и на документирање на влијанието на промените во околината врз истите. Овој нов тренд на истражувања може да се примени на *Bacillus* spp. од почва во РС Македонија во трите климатски типови поради разликите помеѓу нив. Ова истражување се фокусира на определување на геохемиските параметри на почвата и микробиолошки анализи. Беа изолирани вкупно 36 соеви на *Bacillus* spp., а 5 од нив покажаа антимикробна активност врз одредени тест микроорганизми. Според добиените резултати исто така беше детерминирано дека разновидноста на видовите на *Bacillus* се менува според микросредината на почвата под влијание на различни климатски услови.

Клучни зборови: *почвени бактерии, климатски промени, земјоделство, антибиотици, пробиотици.*