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Isolation of soil microorganisms and their potential applications against phytopathogenic fungi

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Abstract

A contemporary substitute that shields the environment from the widespread issue of pesticide pollution includes biofungicides, natural, environmentally friendly, and biodegradable pest management solutions that are targeted at specific pest species and do not induce resistance in their intended targets. The Republic of North Macedonia, is still reliant on imports of these bioproducts until research is done on the inherent potential of its soil micro-communities. The purpose of this study was to isolate native microorganisms that show antifungal activity, as biofungicides are ecological bioproducts. The results showed that 80% of the 116 of isolates were from contaminated soils. The highest number of bacteria was found in Rudarsko energetski-kombinat-Bitola (REK) (15.67 x 10^4 $CFUg^{-1}$) in the summer season and the lowest number in Bucim (2.8 x 10^2 $CFUg^{-1}$) in winter. From the results obtained during the research, it can be noted that the number of microorganisms showed great seasonal variations and was influenced by the soil type. Out of 116 bacterial isolates, 45 exhibited antifungal activity against *Monilinia fructicola*, *Aspergillus niger* and *Penicillium* sp. *in vitro*. Isolates from contaminated soils showed greater antifungal activity. Antagonists B85, B86, B87 and B88 stood out by forming the largest zones of inhibition against phytopathogenic fungi. The collected test results show that four weeks after applying the individual isolates and its consortium, there was a drop in the number of phytopathogenic fungus in the soil-filled pots. Thus, it can be concluded that the obtained results can be used for further research in order to apply these microorganisms as biological control preparations, a source of green chemicals, as biostimulators for improving soil fertility, encouraging plant growth and reducing of pesticide toxicity.

Keywords: soil microorganisms, antifungal activity, phytopathogenic fungi, microbial biofungicides

Introduction

„Specific preparations containing living microorganisms“ is a precise definition of „biofungicides,“ but more broadly, it can refer to botanical substances, semiochemicals (such pheromones), and transgenic products. (Regnault-Roger & Philogène 2008; Liu et al. 2021). In particular, bioformulated products made from beneficial microorganisms (fungi, yeast, bacteria, and actinomycetes) or their metabolites are becoming more and more appealing as plant protection products for both standalone applications and the Integrated Pest Management (IPM) in the modern agricultural system (Fontenelle et al. 2011; Singh et al. 2011; Harman et al. 2012; Małolepsza et al. 2017; Liu et al. 2021). Nowadays, the most effective way to manage bacterial and fungal phytopathogens is to use synthetic fungicides and bactericides (Heydari & Pessarakli, 2010). Fortunately, microbial control of plant diseases and the use of their derivatives to create biopesticides provide us the opportunity to replace the chemicals with more sustainable and environmentally acceptable

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alternatives (Khalili et al. 2016; Gao et al. 2018; Wallace et al. 2018; Raymaekers et al. 2020). Following the first green revolution, which primarily decreased the indiscriminate use and adverse consequences of chemical pesticides, the idea of biopesticides was introduced. Crop management techniques that take advantage of a variety of soil and rhizospheric microorganisms to function as plant biostimulators are both less hazardous and more sensible (Muhammad et al. 2022). Antagonistic microbes aid in preventing the growth of phytopathogens (Brotman et al. 2012; Chauhan et al. 2016).

According to Cavalcanti et al. (2006), soil is one of the best environments for microbial growth, which is why isolated soil microorganisms are leaders in this field. Strong anthropogenic influences on soil, such as heavy metal and ecotoxic chemical contamination, result in degraded ecosystems that are especially important for understanding microbial ecology and human health (Yilmaz et al. 2005). These ecosystems exhibit a range of responses to the added contaminants, including modifications to the biodiversity's qualitative composition and adjustments to population dynamics, particularly pertaining to microbes (Gebreel et al. 2008). Since the microbiota reacts to these pollutants first, it is common practice to look for bacteria with potential for suppressing phytopathogenic fungi among the bacterial and fungal populations found in contaminated soils. These conditions are exerting selection pressure on a wide range of organisms, including bacteria that have rapidly adapted to these environments (Gaete et al. 2020). Importantly, the industrial effectiveness of biocides is attributed to their non-toxic processes, which allow the fungi/bacteria to parasitize and suppress other harmful microorganisms. Since then, bioformulated solutions have made significant strides toward a more environmentally friendly agronomic pest management strategy (Muhammad et al. 2022).

By using this concept, we hope to take advantage of the microbial members of these contaminated communities' innate mechanisms of antibiosis and to study this well-known occurrence at the soil level in the Republic of North Macedonia. At the moment, the Republic of North Macedonia imports the majority of these goods from the USA, the Netherlands, and Bulgaria. Prior to beginning the manufacture of our Macedonian biopesticides, we must recognize the inherent potential of our micro-communities. The introduction of such products to the national market will unavoidably lessen the reliance on traditional chemical fungicides, which would have a direct impact on the amount of pesticide contamination in the Republic of North Macedonia and provide a path toward a more environmentally friendly future. The aim of this study was to isolate soil microorganisms and determine their antifungal activity against phytopathogenic fungi.

Materials and methods

Soil sampling

Samples were collected from different locations in the Republic of North Macedonia (REK, Bucim, Ohis and Mount Baba). Soil samples were collected in the winter, spring, summer and autumn during 2023. The selection took into account conservation, availability of the sample site and anthropogenic disturbances (proximity

Figure 1. Soil sample collection

to a forest road, etc.). In the process of sampling, the local temperature as well as the associated geographic data (altitude, coordinates) were noted. One meter by one meter wooden experimental plots were used to gather the material in the field. 15 to 20 cm below the surface, samples were taken. Using a sterile spatula, soil was gathered and then brought in sterile bags within an ice-filled refrigerator box to the Department of Microbiology and Microbial Biotechnology laboratory at the Faculty of Natural Sciences and Mathematics. The laboratory was accessed a day later. (Figure 1).

Determination of soil moisture content and pH

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. Twenty grams 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.

Isolation and enumeration of bacteria

To determine the number of bacteria 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. Five millilitres of each was put into sterile, empty test tubes after the bottle had been thoroughly mixed. Using the pour plate method, each of the dilutions (10^{-1}-10^{-8}) was infected into a nutritional agar plate (NA). Following 24 and 48 hours of incubation at 37 °C, the bacterial growth on the inoculation plates was observed. The number of bacterial colonies (CFUg^{-1} – (Number of colonies*dilution factor) / volume of culture plate) represented as CFUg^{-1} of the original sample was used to conduct quantitative assessments of the bacterial growth in the samples. The morphological traits of the bacteria dictated qualitative analyses. The colony‘s morphological shape and color were then described for this purpose by observing the macroscopic features. Prior to starting the microscopic study, the streak method was used to separate pure cultures from the previously inoculated plates, and the Gram stain method was used to prepare microscopic slides. A microscope with a 100× objective was used for the microscopic inspection.

Determination of antifungal activity in vitro

The determination of the antifungal activity of the isolates against the test microorganisms was conducted using the agar well diffusion method. The test fungi (*Aspergillus niger* ATCC 16404, *Monilinia fructicola* and *Penicillium* sp.) used in this analysis were isolated from bread and apricot by observing the macroscopic features, and included in the microorganism collection of the Microbiology Laboratory at the Faculty of Natural Sciences and Mathematics, Skopje, North Macedonia. All test fungi were inoculated in Sabourad-dextrose broth (SDB) and incubated at 25°C for 72 hours. The fungal strains were inoculated onto each sterile nutrient agar (NA) Petri dish using a sterile swab, and then wells with a diameter of 8 mm were punched in the plates using a sterile stainless steel borer. Into these wells, 20 µL of the isolates which were allowed to pre-incubate at 37°C for 24 hours in tubes with nutrient broth without shaking, were added. The plates thus inoculated were incubated at 37°C for 24 hours, and then the diameter of the zone of inhibition, expressed in mm was measured for each isolate.

Monitoring of the biofungicide in semi-laboratory conditions

The four selected isolates were further multiplied on a shaker at 180 rpm for 48 hours at room temperature after being seeded in Erlenmeyer flasks containing 50 ml of nutrient broth. The isolates were centrifuged at 4000 rpm for fifteen minutes. Following centrifugation, each isolate‘s biomass was transferred to a sterile Erlenmeyer flask, where 1 ml of the isolates was mixed, using saline as the solution, with final concentration of 10^8 CFUs ml^{-1}. The preparation was applied to the previously sterilized compost soil in autoclave. The soil was divided into two containers: one was used as a control (containing fungus but no preparation), and the other was used as a test (containing fungus and preparation), using triplicates. To the soil pots, a 10^8 CFUs ml^{-1} of *Monilinia fructicola* was added. For thirty days, the pots were incubated at room temperature. Ten grams were extracted from each pot after 7, 14, 21 and 28 days in order to measure the amount of fungus present in the soil, as described above.

Results

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

Following the determination of the moisture content of each type of soil in each of the four seasons, the soil from the Ohis area had the highest value in the winter and the soil from Bucim had the lowest value (Table 2). When the pH of each type of soil was measured, the Ohis soil had the greatest pH value (7.82) in the summer, and the Mount Baba soil had the lowest pH value (5.93) in the winter (Table 3).

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

Location	Coordinates	Altitude	Temperature (winter)	Temperature (spring)	Temperature (summer)	Temperature (autumn)	Ecosystem
Mount Baba	N41°02'01.176" E21°13'34.6152"	1400 m	13 °C	17 °C	10 °C	2 °C	Coniferous forest (undisturbed soil)
REK	N41°03'08.226" E21°28'22.7748"	632 m	8 °C	32 °C	20 °C	10 °C	Urban ecosystem (contaminated soil)
Ohis	N41°39'10.068" E22°21'22.5792"	616 m	21 °C	35 °C	25 °C	4 °C	Urban ecosystem (contaminated soil)
Bucim	N41°57'34.5528" E21°29'15.0792"	277 m	7 °C	34 °C	22 °C	5 °C	Urban ecosystem (contaminated soil)

Table 2. Moisture content (%) for each soil type

Season	Mount Baba	REK	Ohis	Bucim
Winter	19%	20%	21.3%	8.5%
Spring	11.65%	11.65%	21.65%	1.65%
Summer	11.3%	9.9%	11.25%	0.95%
Autumn	1.76%	3.66%	4.81%	1.81%

Table 3. pH value for each soil type

Season	Mount Baba	REK	Ohis	Bucim
Winter	5.93	7.80	7.70	7.58
Spring	6.24	6.60	7.63	7.31
Summer	6.66	6.93	7.82	7.48
Autumn	5.95	6.98	7.42	7.11

The results from determining the number of bacteria are shown in Figure 2. According to the obtained results, the highest number was recorded in REK (15.67 x 10^4 $CFUg^{-1}$) in the summer season and the lowest number in Bucim (2.8 x 10^2 $CFUg^{-1}$) in winter.

Screening of antifungal activity of the 116 isolates was performed against 3 test fungal isolates. The number of isolates shown in table 4 were found to have inhibitory activity against at least one test microorganism (Table 4). Out of 45 isolates that showed antifungal activity, 21 showed inhibitory effect against only one fundal isolate, 17 against 2 fungal isolates and 7 isolates against the three tested fungal isolates. The isolate B40 showed the smallest inhibition zone (12.67 mm) against *Aspergillus niger* ATCC 16404, while the isolate B112 showed the largest inhibition zone (23.88 mm) against *Monilinia fructicola*.

Four isolates B85, 86, 87 and 88 that showed broad antifungal activity towards all three fungal isolates were selected for soil applications in semi-controlled laboratory conditions. (Figure 3).

The collected test results show that four weeks after applying the isolates, there was a drop in the number of

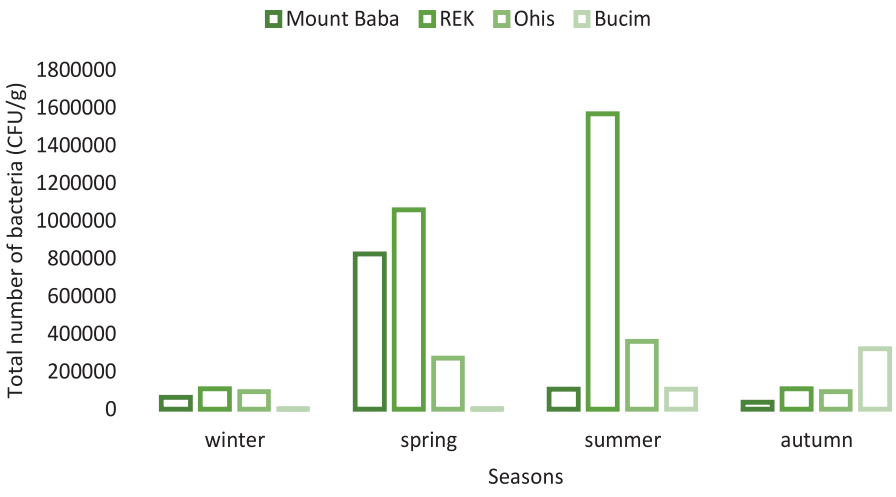

Figure 2. Number of total bacteria ($CFUg^{-1}$) from each soil type in four seasons during 2023

Table 4. Zones of inhibition (mm) of the indicator fungi caused by bacterial isolates

Isolate	*Monilinia fructicola*	*Penicillium* sp.	*Aspergillus niger* ATCC 16404
B13	/	/	15.85
B14	/	15.43	/
B17	/	18.95	17.79
B18	/	18.36	18.95
B19	/	18.04	19.33
B20	/	19.79	18.72
B22	/	17.86	/
B24	/	18.00	/
B26	/	/	17.20
B28	15.76	/	/
B29	/	16.90	/
B30	/	16.80	/
B32	/	14.52	/
B33	/	16.26	/
B34	/	15.51	/
B38	18.25	16.40	16.38
B39	13.05	/	18.05
B40	/	/	12.67
B43	/	/	15.11
B44	13.9	/	/
B45	/	13.78	15.43
B46	/	/	12.02
B47	/	17.35	/
B49	/	14.08	/
B56	/	/	15.32
B60	18.76	21.32	16.28
B63	15.71	20.81	14.57
B65	18.08	18.73	18.77
B71	16.84	15.31	/
B72	14.85	19.02	/
B74	/	15.54	14.08
B81	18.28	/	/
B85	/	15.09	16.63
B86	15.65	14.87	17.45
B87	17.77	17.80	17.08
B88	17.08	19.61	17.67
B93	/	15.85	17.55
B94	/	17.27	13.23
B98	14.09	15.83	/
B103	16.78	17.21	/
B107	/	17.72	/
B110	15.77	22.48	/
B112	23.88	23.49	/
B115	15.69	18.33	/
B116	/	20.82	/

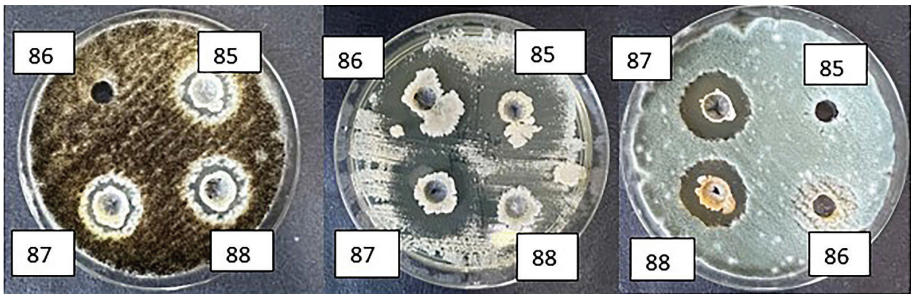

Figure 3. Zones of inhibition of the isolates B85, B86, B87, B88 against *Aspergillus niger* ATCC 16404 (left), *Monilinia fructicola* (middle) and *Penicullium* sp.

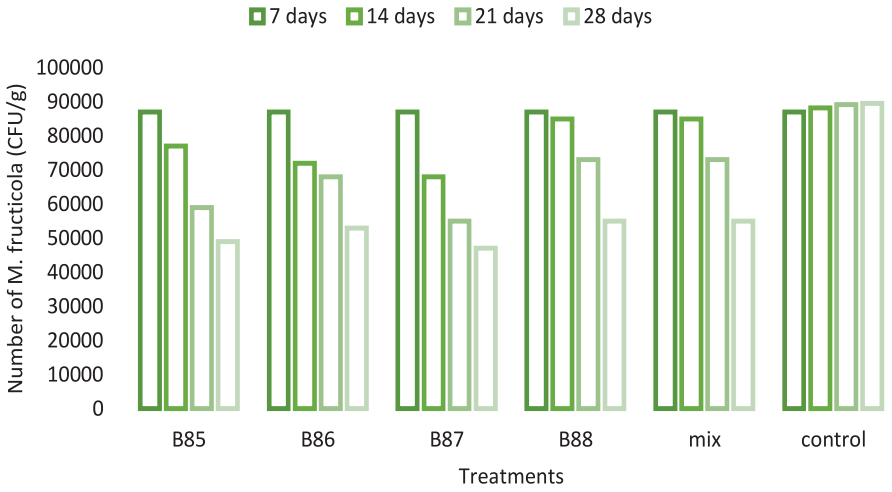

Figure 4. Number of *Monilinia fructicola* (CFUg^{-1}) within four weeks after applying the isolates

M. fructicola in the soil-filled pots. The soil inoculated with a mixture of isolates showed the largest difference compared to the non-treated control, indicating that the consortium of bacteria has a stronger effect on lowering the amount of phytopathogenic fungus than individual bacterial strains (Figure 4).

Discussion

With regard to the findings about the soil moisture content, all soil samples show a tendency of decreasing moisture percentage from the winter to the summer. The most likely cause of these seasonal variations in moisture content is outside factors like rainfall and temperature. Because of this, soil moisture content is low throughout the summer, when temperatures are high and rain is infrequent. The research's findings validate the impact of outside factors on moisture content. The buildup of chemicals in the pores between soil particles may be the cause of the contaminated soil's low moisture content, which could lead to a decrease in the soil's ability to absorb water and oxygen (Osam et al. 2013). According to this study's findings, pH is more dependent on organic matter, soil chemistry and texture, and decomposition processes. Through their impacts on soil organic carbon concentration, precipitation and temperature can influence changes in soil pH. A high value of this concentration can lead to low soil pH because it can produce more organic acid and have stronger H^+ sorption (Deng et al. 2014).

Seasonal differences in abundance of bacteria led to the conclusion that temperature changes have a significant impact on abundance. With the exception of REK, it is evident from the results that the abundance is higher in the spring and autumn than it is in the summer and winter. Temperature is one of several abiotic elements that influence the microbial population in the soil; it influences other sub-factors that make up the soil microbiome independent of anthropogenic and climatic changes. This method is becoming equally important for examining the structure and diversity of the soil microbial community at various climatic and geographic scales, as evidenced by multiple research examining seasonal fluctuations in the bacterial community profile of alpine forest soils (Philippot et al. 2007).

In the Republic of North Macedonia, the variation in soil microbe abundance has received somewhat

less research and this study aims to examine how the temperature has affected the microbial population in the soil over time (Lafleur et al. 2005). The results show that there is a considerable variation in the number of microorganisms, suggesting that temperature affects the microbial population in various soil types. This study, which has never been published in the literature, demonstrates the variation in the quantity of microorganisms isolated from various soil types in the Republic of North Macedonia, at different periods, despite the same laboratory growing conditions. The relationship between the microbial population and other ecological features in the Republic of North Macedonia's territory which could suffer disastrous consequences from urbanization and climate change needs further investigation.

Plant diseases are currently able to be controlled efficently by synthetic chemicals, but their severity can be reduced by using plant protection techniques. Owing to the adverse impacts of synthetic pesticides and the growing resistance of pathogens in ecosystems, a new biological strategy that promotes environmentally safe practices and sustainable agriculture should be employed (Azizbekyan 2019). A secondary metabolite that was isolated from *Bacillus subtilis* HussainT-AMU was found to have a highly significant effect against the pathogen *Rhizoctonia solani* in a research. Its use was also associated with good yield and plant growth. The isolated metabolite shared structural similarities with surfactin, a lipopeptide. The findings of this study supported the potential of *Bacillus subtilis* HussainT-AMU for future use as a biosurfactant producer and the introduction of novel biocontrol techniques, as well as its capacity to control the pathogen *Rhizoctonia solani* in an environmentally friendly manner (Hussain & Khan 2019).

Through soil isolation, the bulk of *Bacillus* sp. strains that are fungus-hostile have been found. Research has substantiated the noteworthy role of *B. subtilis* and *B. amylobacillus* as *M. fructicola* antagonists (Yuan et al. 2019). *B. subtilis* produces a wide range of antimicrobial chemicals, including as fengycin, iturin, and surfactin, which are antagonistic to *M. fructicola* (Yánez-Mendizábal et al. 2012; Mnif et al. 2016). The findings of another study, by using molecular and physicochemical techniques, identified the bacillus XJ-C strain as *B. methylotrophicus*. It was discovered that this bacterium strain had an antagonistic effect on *M. fructicola* through *in vitro* experiments conducted on peach fruit, leaves, and shoots (Yuan et al. 2019).

In this regard, we might strive toward the creation of biofungicides that serve as ecological products through additional *in vitro* and *in situ* research. In contrast to chemical pesticides, biofungicides are natural, safe for the environment, and biodegradable solutions that kill pests without creating resistance in their intended targets (Muhammad et al. 2022). The incidence of environmental pollution is expected to decrease when biofungicides replace chemical pesticides (Montesinos 2003).

Conclusion

This study showed that 45 out of 116 isolates have strong *in vitro* antagonistic activity against fungal pathogens. According to the results, it was found that the number of soil microorganisms changes depending on the different climatic conditions. Since 80% of the isolates were from the contaminated soil samples it is standard procedure to search for bacteria with potential for suppressing phytopathogenic fungi among the bacterial and fungal populations found in contaminated soils given that the microbiota responds to these pollutants first. The majority of these antimicrobial activity mechanisms are group-specific and have the potential to be used in the management of other pathogenic or dangerous organisms that were not the intended target of the antimicrobial adaptations found in contaminated soil members. In addition, the territory of the Republic of North Macedonia contains a large number of contaminated soils from the heavy, chemical and mining industries. By applying this approach, it is possible to take advantage of the microbial members of these contaminated communities' innate mechanisms of antibiosis, and at the same time, this well-known phenomena may be seen at the soil level in the Republic of North Macedonia. Prior studies have demonstrated that soil microorganisms can impede the development of pathogenic fungus by secreting one or more antimicrobial substances, including antibiotics and antimicrobial peptides. Antifungal strains will undoubtedly yield an increasing number of antimicrobial compounds for use in agricultural production as long as antimicrobial compounds are researched and extracted.

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